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VOL. 10 . MARCH 1950 No. 3

CONTENTS

Simon Koletsky, Frederick J. Bonte, and Hymer L. Friedell. Production of Malignant Tumors in Rats with Radioactive Phosphorus 129

Eric Ponder, Heat Coagulation of Serum in Cancer: A Method Applicable to Very Small Quantities of Serum 139

Gail Lorenz Miller, Elizabeth Ufford Green, Joseph J. Kolb, and Elizabeth Eshelman Miller. Studies on the Proteins of Rhabdomyosarcoma and Normal Muscle of Mice. I. Gross Composition, Extractions with 0.5 M KCl, and Fractionations by Differential Centrifugation and Dialysis 141

Gail Lorenz Miller, Elizabeth Ufford Green, Elizabeth Eshelman Miller, and Joseph J. Kolb. Studies on the Proteins of Rhabdomyosarcoma and Normal Muscle of Mice. II. Electrophoretic and Viscometric Measurements 148

Charles D. Stevens, Mary Ann Meinken, Patricia M. Quinlin, and Paul H. Stewart. The Distribution of Radioactive Iodine in Mice with and without Tumor 15091a after Injection of Radioactive Sodium Iodide 155

Min Hsin Li and W. U. Gardner. Influence of Age of Host and Ovaries on Tumorigenesis in Intrasplenic and Intrapancreatic Ovarian Grafts 162

Howard E. Skipper, Leonard L. Bennett, Jr., Pradisth C. Edwards, Carl E. Bryan, Olivia S. Hutchison, Juanita B. Chapman, and Martelia Bell. Anti-leukemic Assays on Certain Pyrimidines, Purines, Benzimidazoles, and Related Compounds 166

Alfred Gellhorn, Morris Engelman, Daniel Shapiro, Samuel Graff, and Horace Gillespie. The Effect of 5-Amino-7-Hydroxy-1*H*-*v*-Triazolo (*d*) Pyrimidine (Guanazolo) on a Variety of Neoplasms in Experimental Animals 170

Kanematsu Sugiura, George H. Hitchings, Liebe F. Cavalieri, and C. Chester Stock. The Effect of 8-Azaguanine on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma and Melanoma in Animals 178

L. W. Law. Studies on the Effects of a Guanine Analog on Acute Lymphoid Leukemias of Mice . 186

James B. Murphy and Ernest Sturm. The Effect of Adrenal Grafting on Transplanted Lymphatic Leukemia in Rats 191

Lew Cunningham, A. Clark Griffin, and J. Murray Luck. Effect of a Carcinogenic Azo Dye on Liver Cell Structure. Isolation of Nuclei and Cytoplasmic Granules 194

Announcement 200

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CANCER RESEARCH

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Production of Malignant Tumors in Rats with Radioactive Phosphorus*

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The development of malignant neoplasms in rats has been observed in the course of studies on the late effects of radioactive phosphorus poisoning. Of thirteen tumors, ten were osteogenic sarcoma and three were squamous-cell carcinoma. Data pertinent to the carcinogenic action of radiophosphorus will be presented.

PROCEDURE AND RESULTS

Neoplasms were observed in two experimental groups of rats, i.e., (*a*) animals surviving a single LD_{50} dose of P^{32} (4.5 μc. per gram of body weight), and (*b*) those surviving repeated injections of P^{32} (1.5 μc. per gram of body weight every 3 weeks) for a total dose of 9 or 12 μc. per gram. Adult white male rats of the C. F. Wistar strain, which weighed about 150 gm. each, obtained from Carworth Farms, were used. Spontaneous bone tumors have not been observed among normal animals of this strain (1). The rats were kept in an air-conditioned unit permitting constant temperature and humidity. Radioactive material was injected by the intraperitoneal route.

Complete autopsies were performed on all rats which died or were sacrificed. In almost all animals routine microscopic sections of femur and vertebra were taken. X-rays were taken of the entire skeleton of most animals, usually at the time of death or sacrifice.

For controls, x-rays were made of the skeleton of: (*a*) twenty normal adult animals of the C. F. Wistar strain, maintained for the same time and under the same conditions as the experimental rats, and (*b*) eight rats each of which had received, intraperitoneally, a dose of 1 mc. of radioactive iodine^{131} 1 year previously. None of these showed evidence of bone neoplasm.

GROUP 1.—In this laboratory 4.5 μc. of P^{32} per gram of body weight is approximately the LD_{50} dose. Rats injected with this amount usually died within 3 weeks (20-day LD_{50}) and at autopsy showed marked aplasia of bone marrow and involution of lymphoid tissues. Many of the surviving animals were set aside for extended observation. The object was to determine, in relation to time, the extent of repair in organs severely damaged by a single dose of internal radiation: especially the hemopoietic system, the presence of irreversible tissue lesions; and, finally, any late effects of the P^{32}. To date, study has been completed on nineteen such rats, sixteen of which were sacrificed from 6 months to 1 year later, while three died, i.e., one at 7 months and two at 9 months after administration of radiophosphorus.

Nine rats in this group developed malignant neoplasm. The animals were otherwise in good condition and had gained weight over the experimental period, except in the last few weeks of life when there was progressive anorexia and emaciation.

Rat 56.—This rat died 7 months after injection of the P^{32}. During the last 2 weeks of life, there was progressive swelling of the right side of the face. Autopsy revealed an extensive tumor involving

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right maxillary and premaxillary bones. There was invasion and destruction of the roof of the mouth, also extension into the soft tissues of the face. Microscopically, the neoplasm was an osteogenic sarcoma. No metastasis was demonstrated.

Rat 59.—This rat was sacrificed 5 months after receiving an LD_{50} injection of radioactive phosphorus. The anterior and right lateral aspects of the face were expanded, covered by thick, nodular, grayish-red skin, and there was marked exophthalmos of the right eye (Fig. 1). X-ray showed destruction of the right zygoma and posterior portion of the right maxillary bone, and also possible involvement of the right ethmoid and temporal bones (Fig. 2). A section revealed a massive, moderately firm, grayish-white tumor, involving and partly destroying the right nasal, ethmoid, zygomatic, and maxillary bones. The neoplasm infiltrated the soft tissues of the face widely, and involved skin, roof-of-mouth, naso-pharyngeal passage, Harderian gland, ethmoid sinus, and the right olfactory bulb of brain. Superficial and deep cervical lymph nodes were greatly enlarged, measuring up to 1 cm. in diameter. Microscropic study

FIG. 1 (Rat 59).—Face of rat with squamous-cell carcinoma, probably arising from the lining of nasal sinus.

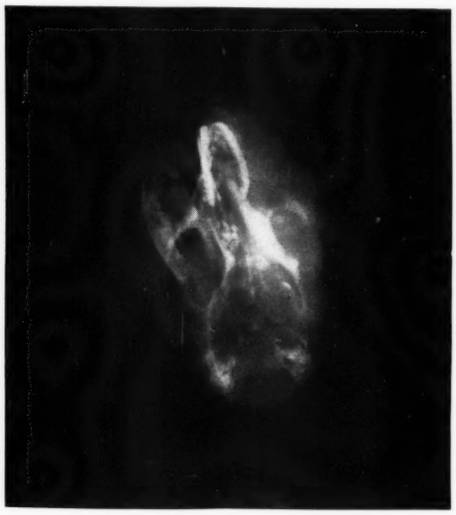

FIG. 2 (Rat 59).—X-ray of skull showing destruction by tumor of right zygomatic arch and maxillary bone.

FIG. 3 (Rat 59).—Cervical lymph node, H & E × 86. Metastatic well differentiated squamous-cell carcinoma

revealed a well-differentiated squamous-cell carcinoma with the mouth, naso-pharynx, or epithelial lining of the ethmoid sinus as possible sites of origin. There were metastases to the right cervical lymph node chain (Fig. 3).

Rat 53.—This rat died 9 months after administration of the P^{32}. Terminally, there was anorexia and weight loss. An extensive lesion, probably an osteogenic sarcoma, was found, which was evidently neoplastic and involved the right maxillary bone and roof of the mouth. The latter was necrotic, ulcerated, and largely destroyed. This animal was discarded by accident, and microscopic confirmation of the tumor could not be made.

Rat 187.—This rat was sacrificed 8 months after receiving an LD_{50} dose of P^{32}. The animal was otherwise in good condition and showed no clinical evidence of tumor. X-ray at the time of sacrifice showed irregular sclerosis of the proximal diaphysis of the right tibia, with bone formation and extension into the surrounding soft tissues (Fig. 4). Microscopic study revealed an invasive osteogenic sarcoma, involving the periosteum, cortex, and cancellous part of the bone. There were several metastatic nodules in the lungs and within the lumens of pulmonary blood vessels (Fig. 5).

Rat 57.—This rat was sacrificed when in poor condition 1 year after receiving radiophosphorus in LD_{50} dose. Several weeks before death, the animal showed atrophy and paralysis of the left leg, which was inwardly rotated and fixed. X-ray of the pelvis at the time of death disclosed a large well-defined mass, arising from the wing of the left ilium. There were numerous calcified areas at the center. The ilium was partially destroyed (Fig. 6). At autopsy a hard mass, 4 cm. in diameter, was

FIG. 4 (Rat 187).—A-P and lateral films showing a destructive and invasive tumor of the proximal diaphysis of the right femur.

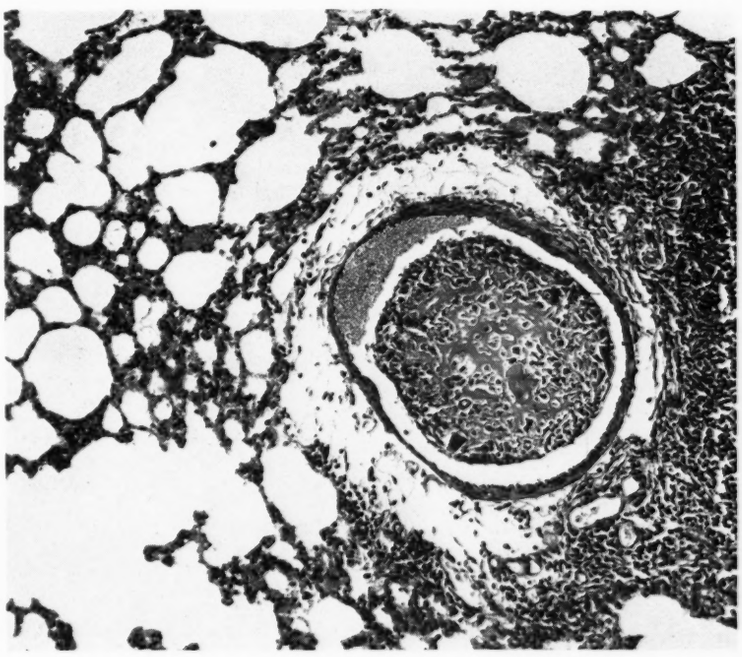

FIG. 5 (Rat 187).—Lung, H & E × 86. Nodule of osteogenic sarcoma within lumen of pulmonary blood vessel

observed, which was attached along the entire pelvic aspect of the left ilium and which protruded into the abdominal cavity, filling most of the entire left lower quadrant (Fig. 7). The sigmoid colon was displaced to the right by the neoplasm, which extended up to the inferior pole of the left kidney, compressed the left iliac vessels and nerves, and was attached to the oblique abdominal muscles. The outer and cut surfaces were hard, bony in consistency, grayish-white or pink, nodular, and richly vascularized. Microscopically, the tumor was a typical osteogenic sarcoma, and there were multiple metastatic nodules in the lungs (Fig. 8).

FIG. 6 (Rat 57).—X-ray of pelvis. Large tumor mass attached to left ilium

Rat 188.—This rat was sacrificed 10 months after receiving radiophosphorus. For several weeks prior to death there was progressive swelling and protrusion of the anterior and lateral aspects of face. Films of the head, at the time of sacrifice, showed a soft tissue swelling of the face containing small areas of calcific density. The tumor extended to the right premaxillary bone (Fig. 9). At autopsy a large, hard, grayish-white, bony tumor was observed attached to the periosteal surface of the right nasal and premaxillary bones, compressing the right nasal cavity, widely infiltrating the soft tissues of face, and extending to the skin. The neoplasm was an osteogenic sarcoma. Microscropic sections revealed metastasis to the lungs.

Rat 72.—This rat was sacrificed 1 year after injection of the P^{32}. The animal was in fairly

FIG. 7 (Rat 57).—The neoplasm of left ilium extends into the abdominal cavity.

good condition, despite moderate weight loss over the last 2 months. Exophthalmos of the right eye was noted a few weeks before sacrifice. X-ray findings of the head and the rest of the skeleton were negative. Autopsy revealed an infiltrating well-differentiated squamous-cell carcinoma of the soft tissues beneath the right eye, with involvement of the Harderian gland and extension to the ethmoid sinus. The precise site of origin was not evident. No metastasis was found.

Rat 159.—This rat was sacrificed 1 year after receiving an LD_{50} dose of P^{32}. Condition of the animal was fair, although marked left exophthalmos had been observed about 2 weeks before sacrifice. X-ray findings of the head were negative. Autopsy showed an extensive ulcerating tumor of the roof of the mouth extending laterally to the left mandible and also invading the Harderian gland. The neoplasm was a well-differentiated squamous-cell carcinoma. There was no demonstrable metastasis.

Rat 197.—This rat was sacrificed 11 months after receiving P^{32}. One week before death, paralysis of the right fore limb was noticed, and also weakness of the left fore limb and of both hind extremities. X-ray showed a sclerosing neoplasm of the second cervical vertebra (Fig. 10). At autopsy a hard white neoplasm was noted, 1 cm. in diameter, involving C_2, invading the spinal canal, and compressing the cord (Fig. 11). The neoplasm was an osteogenic sarcoma. No metastasis was found.

GROUP 2.—In order to observe the tolerance of rats to large, repeated doses of radiophosphorus, a preliminary experiment was carried out, using fifteen rats. The schedule called for eight successive intraperitoneal injections, each of 1.5 μc. per gram body weight of the radioactive material, at 3-week intervals. This was a total of 12 μc. per gram, almost three times the single LD_{50} dose.

One rat was dead 10 days after injection of the first dose of radiophosphorus; one died after two doses, one after four doses, one after five doses, two after six doses, and one after seven doses.

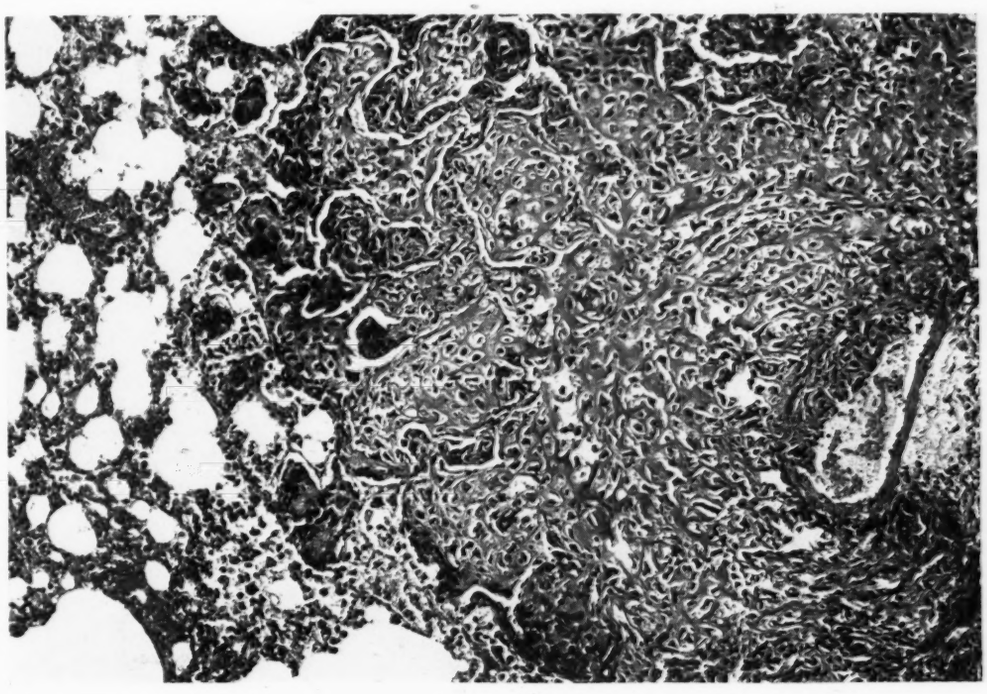

FIG. 8 (Rat 57).—Lung, H & E × 86. Metastatic osteogenic sarcoma, showing extensive intercellular matrix of bone

FIG. 9 (Rat 188).—Film of head showing soft-tissue swelling of face containing small areas of calcific density.

FIG. 10 (Rat 197).—Film of spine showing sclerosing neoplasm of the second cervical vertebra.

FIG. 11 (Rat 197).—Tumor mass attached to the right side of the second cervical vertebra.

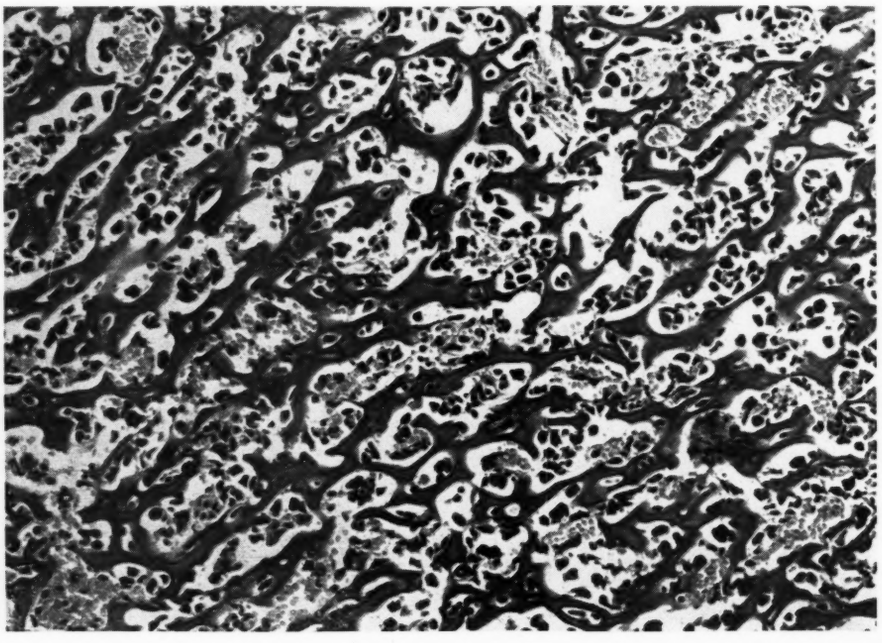

FIG. 12 (Rat 225).—Osteogenic sarcoma of jaw, H & E × 186. There is pleomorphism of tumor cells and abundant formation of bone.

Eight of the fifteen rats survived all eight doses; of these, three died at intervals of a few to several weeks after injection of the eighth dose, while the remaining five were sacrificed from 1 to 2 months after receiving the eighth dose.

Complete autopsies were performed on eleven of the fifteen rats. Four animals were either discarded accidentally after death or showed too advanced autolysis for study.

Three rats in this group developed a malignant tumor of bone which was evident in the gross, while a fourth showed a probable early osteogenic sarcoma of the femur, diagnosed microscopically. All four animals showed progressive weakness, anorexia, and weight loss over periods from 2 to 6 weeks before death or sacrifice. Prior to this terminal phase, the weights had increased or were maintained.

Rat 225.—This rat died 2 weeks after receiving the sixth successive dose of P^{32}, i.e., 4 months after the onset of the experimental period. Total dosage was 9 μc. per gram. There was an extensive, destructive, and expanding neoplasm of the right maxillary bone involving the roof of the mouth. The tumor infiltrated the adjacent soft tissues widely, forming a large, hard, grayish-white mass. Microscopic examination showed an osteogenic sarcoma (Fig. 12). No metastasis was found.

Rat 226.—This rat was sacrificed when in poor condition at 6 months, 1 month after injection of the eighth successive dose of P^{32}. The total amount received was 12 microcuries per gram. Films at this time showed involvement of the proximal one-fourth of the right tibia by a destructive and invasive lesion. The latter formed a large mass with irregular calcific density (Fig. 13). Autopsy revealed an osteogenic sarcoma (Fig. 14) involving metaphysis and adjacent shaft of bone and infiltrating the soft tissues. Metastasis to the lungs was demonstrated microscopically.

FIG. 13 (Rat 226).—Film showing destructive neoplasm of the proximal fourth of the right tibia with invasion of soft tissues.

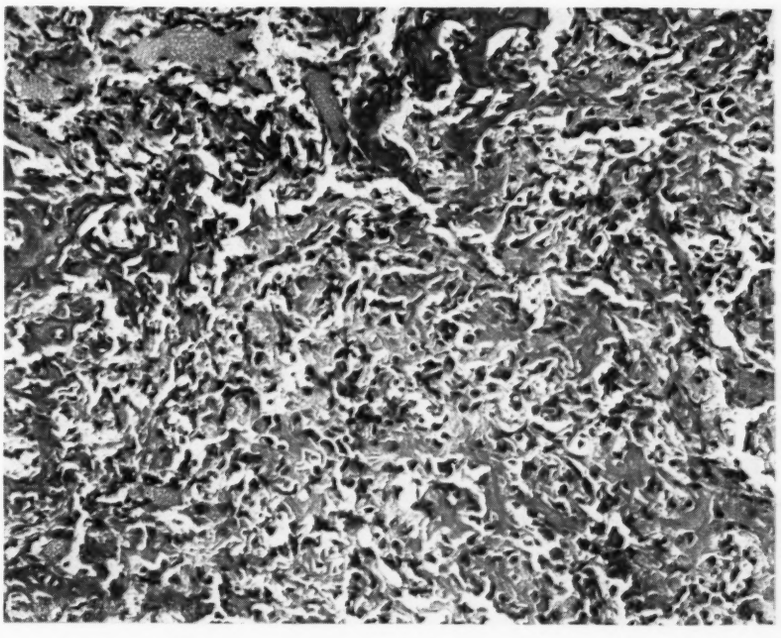

FIG. 14 (Rat 226).—Right tibia, H & E × 134. Osteogenic sarcoma with prominent intercellular bony matrix

Rat 298.—This rat was sacrificed when in poor condition at $6\frac{1}{2}$ months, 6 weeks after administration of the eighth dose of radiophosphorus. Two weeks before death, enlargement of the lower ab-

domen (cord bladder) and complete paralysis of the hind extremities was noted. Antero-posterior and lateral x-rays showed a decrease in density of the body and neural arch of the second lumbar vertebra. The appearance suggested destruction of the vertebra by neoplasm. No accompanying soft-tissue mass was visualized (Fig. 15). At autopsy an extensive osteogenic sarcoma was observed, principally periosteal, but also involving marrow which was the seat of diffuse hemorrhage. There was invasion of the soft tissues, and also encroachment on the spinal canal with compression of the spinal cord. No metastasis was found.

FIG. 15 (Rat 298).—A-P and lateral views of spine showing destruction of the second lumbar vertebra.

Rat 236.—This rat was dead at 5 months, 1 week after the eighth dose of radiophosphorus was injected. There was a lesion, detected microscopically, 2 mm. in diameter, situated in the distal metaphysis of right femur just above the knee, which probably represented an early osteogenic sarcoma. This showed marked proliferation of atypical, irregular or spindle-shaped cells with many mitoses, also pleomorphic and hyperchromatic forms including bizarre giant cells and formation of spicules of atypical bone (Fig. 16).

Morphologically, all the osteogenic sarcomas were essentially similar. In the gross, the tumors varied from firm to hard and were white or grayish-white, solid, and resistant to section. The cut surface usually showed tiny, gritty nodules, evidently of bone.

Microscopically, the tumors consisted of com-

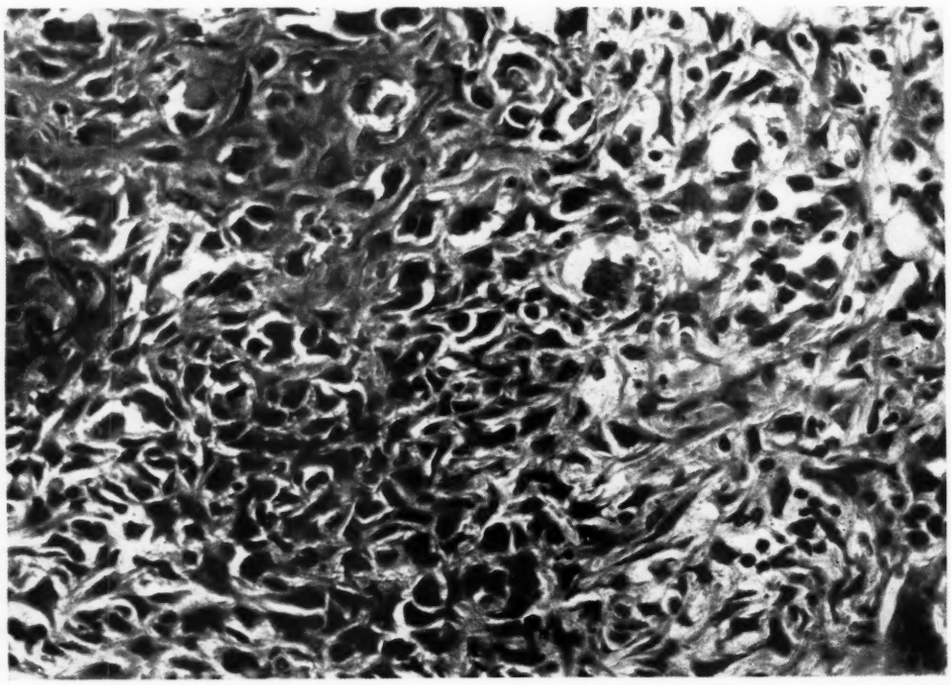

FIG. 16 (Rat 236).—Early osteogenic sarcoma of right femur, H & E × 298. Marked proliferation of atypical cells with pleomorphic and hyperchromatic nuclei, also matrix of atypical bone.

pact groups of highly pleomorphic oval, irregular or spindle-shaped cells with hyperchromatic or vesicular nuclei and poorly defined cytoplasm. There were numerous mitoses. Giant cells were common. An abundant intercellular matrix was present, either of fibrous tissue or bone, and the latter showed an abundance of well-formed trabeculae. Occasionally osteoid or cartilage were found.

SUMMARY OF DATA ON MALIGNANT NEOPLASMS (TABLE 1)

There were thirteen neoplasms, all in adult, white male rats. Three were squamous-cell carcinomas involving the face and were associated with exophthalmos. Ten were osteogenic sarcomas distributed as follows: four in the jaw, two in the spine, two in the tibia, and one each in the femur and pelvis. Four of these tumors metastasized to the lungs.

Incidence.—Malignant tumors occured in nine out of nineteen rats surviving a single dose of P^{32}, an incidence of 47 per cent. This figure is probably low, since most of the rats without neoplasm were sacrificed or died prior to the average latent time for the development of tumor. There were four tumors among the fifteen rats which received repeated injections of radiophosphorus, an incidence of 27 per cent.

Latent period.—With a single dose of P^{32}, the latent period varied from 210 to 360 days and averaged 290 days. With repeated doses of radiophosphorus, the neoplasms occurred from 120 to 200 days and the average was 165 days. These periods indicate detection of the neoplasm, not the exact time of origin. The data suggest that the time of tumor development may be shortened by larger total doses of the radioactive material.

Origin.—The site of origin of the osteogenic sarcomas was difficult to determine. The three tumors of the lower extremities were located in the metaphysis. Of these, two involved both cancellous portion and cortex, including endosteal and periosteal surfaces, while the third was situated mainly within marrow and also involved the endosteum. The neoplasms of ilium and spine appeared to be essentially periosteal.

There were three squamous-cell carcinomas involving the soft tissues of the face. These may have originated from the roof of mouth, nasopharynx, accessory nasal sinuses, or Harderian gland. In these structures the epithelium is closely approximated to bone and was presumably irradiated from this source.

DISCUSSION

This study shows that malignant tumors of bone can be produced experimentally in rats by radioactive phosphorus. Hence, P^{32} is similar in this respect to other radioactive elements such as radium (2, 3), plutonium, cerium[144], yttrium[91], and strontium[89] (4).

In our experiments, the neoplasms occurred in animals which received very high doses of the P^{32}. Whether the carcinogenic property is retained with smaller amounts, and in what proportion, is not known. It is quite probable that the incidence decreases with lower doses in much the same manner as for strontium[89]. Brues, Lisco, and Finkel (5) reported a high incidence of osteogenic sarcoma in mice following single doses of 5 and 2.5 μc. of this isotope per gram of body weight (LD_{50} at 30 days for strontium[89] was 8 μc. per gram body weight) and a considerably smaller incidence with doses of 1, 0.5, and 0.25 μc. per gram. Repeated monthly injections with doses of 1 and 0.5 μc. per gram gave a high incidence for neoplasm, while 0.2 and 0.1 μc. per gram resulted in a much lower incidence.

Radioactive elements such as radium, plutonium, strontium[89], and phosphorus[32] are deposited

TABLE 1

MALIGNANT NEOPLASMS IN RATS FOLLOWING INJECTION OF P^{32}

Rat	Dose of P^{32}	Latent period in days	Type of neoplasm	Location	Metastasis
56	one dose, 4.5 μc per gram	210	osteogenic sarcoma	jaw	none
59	" " " " " "	254	squamous-cell carcinoma	face	cervical nodes
53	" " " " " "	263	not known	jaw	not known
187	" " " " " "	242	osteogenic sarcoma	tibia	lungs
57	" " " " " "	360	osteogenic sarcoma	ilium	lungs
188	" " " " " "	302	osteogenic sarcoma	jaw	lungs
72	" " " " " "	354	squamous-cell carcinoma	face	none
159	" " " " " "	316	squamous-cell carcinoma	face	none
197	" " " " " "	314	osteogenic sarcoma	spine	none
225	repeated doses, total 9 μc per gram	120	osteogenic sarcoma	jaw	none
236	repeated doses, total 12 μc per gram	160	osteogenic sarcoma	femur	none
226	" " " " " " " "	180	osteogenic sarcoma	tibia	lungs
232	" " " " " " " "	200	osteogenic sarcoma	spine	none

heavily in the skeleton, producing radiation damage to bone. The changes have been described under the term "radiation osteitis" (2, 6–8) and consist essentially of degeneration and necrosis of old bone, accompanied by reactive formation of new bone which is usually atypical in arrangement and staining, and often fibrous. Frequently, the new bone also becomes devitalized. The lesions occur in epiphysis and metaphysis, also in the shaft where both endosteal and periosteal surfaces may be involved. The processes of devitalization and formation of atypical bone are similar for various types of radiation (9) (alpha, beta, and gamma ray), apparently with only quantitative differences in the extent of the bone damage which appears due to variation in character and number of the ionizing particles.

In our studies with P^{32}, such bone changes, although varying in degree, were observed almost constantly in femur and vertebrae of rats with or without sarcoma. One lesion, situated at the distal epiphysis of the femur, was sufficiently marked to cause expansion of the bone and was associated with pathological fracture (demonstrated by x-ray). There is strongly presumptive evidence that the lesions serve as a precursor to malignancy, although the precise steps in the metamorphosis are not known (2). If this concept is correct, the neoplasm occurs as part of, or as the end result of, atypical bony growth representing attempted regeneration in regions of radiation necrosis.

The amount of radiation delivered to the skeleton of the rat by an LD_{50} dose of 4.5 μc. of P^{32} per gram was calculated to be about 12,500 reps. This calculation was made by assuming the biological half-life of P^{32} to be 10.3 days. The average skeletal weight of a 200-gm. rat as determined in this laboratory was 13 gm.

The production of malignant neoplasms by P^{32} is of interest in relation to the therapeutic use of this isotope. The doses used in our study are much larger proportionately than those employed clinically. An LD_{50} dose of 4.5 μc. per gram in the rat corresponds to 300 mc. for a 70-kg. human, i.e., about twenty times the usual therapeutic dose. Whether smaller amounts of P^{32} will prove carcinogenic for animals is not known at present. However, very large therapeutic doses of radiophosphorus—and, in fact, of all radioactive isotopes which deposit heavily in bone—would seem to be contra-indicated where long term survival is expected.

SUMMARY

One of the late effects observed in rats injected with large doses of radioactive phosphorus was the development of malignant neoplasms. Of thirteen tumors, ten were osteogenic sarcomas, situated most frequently in the jaw and also occurring in the spine, tibia, femur, or ilium. Four of the neoplasms metastasized to the lungs. There were three squamous-cell carcinomas involving the face and associated with exophthalmos.

The neoplasms occurred in animals which received (*a*) a single LD_{50} dose of P^{32} (average latent period of 290 days) and (*b*) repeated doses of 1.5 μc. per gram of P^{32} (average latent period of 165 days). The incidence of tumor development was about 40 per cent.

Atypical proliferation of bone was usually found in these rats, and the lesions may have served as a precursor to malignant change.

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Heat Coagulation of Serum in Cancer: A Method Applicable to Very Small Quantities of Serum

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The method recently described by Huggins, Miller, and Jensen (1949) for measuring defects in the coagulation mechanism of the sera of cancer patients cannot be applied without modification to the sera of small experimental animals because the quantity of serum required is too large. By substituting a measurement of the opacity of the heated serum for a measurement of its coagulability (or inhibition of coagulation by iodoacetate), values which are well correlated with the iodoacetate index of Huggins, Miller, and Jensen can be obtained; these opacity measurements require a very small amount of serum, and are as applicable to the sera of small animals as to that of man.

Method.—0.05 ml. of serum is added to 0.75 ml. of a NaCl-buffer (four parts of 2.2 per cent NaCl plus one part of a mixture of M/15 NaH_2CO_3 and M/15 Na_2HCO_3) at pH 7.0. The mixture, contained in a small test tube, is placed in a boiling water bath for 30 minutes and is then allowed to cool. The extinction coefficient E of the opaque contents of the tube is found with a microphotometer which has a chamber 1 cm. in depth and has a capacity of about 0.5 ml.; this microphotometer can be constructed by using a microscope tilted horizontally and fitted with a Lange thermopile in place of the eyepiece, together with a potentiometer circuit for measuring the thermopile current. The chamber is fixed on the stage of the microscope in the line of the light path, and the light source is a lamp with a 630 mμ filter. The microphotometer is calibrated by inserting neutral filters with known extinction coefficients in the path of the light, the thermopile current corresponding to each filter being measured; this provides a graph from which potentiometer readings can be translated into extinction coefficients of the opaque diluted serum in the chamber.

The total proteins in the serum are found by a gradient tube density method (2), which requires only a small drop of serum. The extinction coefficient is then divided by the total protein to give the opacity (as a value of E) per gram of protein, a value which bears a relation to the iodoacetate index.

If larger quantities of serum are available, as in the case of man, 0.5 ml. of serum is diluted with 7.5 ml. of the NaCl-buffer and heated; the opacity can then be determined on a macro scale with any one of the types of photometer commonly found in laboratories.

Correlation with iodoacetate index.—The method in which opacity is used in place of coagulability as measure of a change in the protein pattern of serum does not employ iodoacetate as an inhibitor, although the addition of iodoacetate results in a reduction of opacity, on heating, in much the same way as it results in an interference with the coagulation mechanism. To compare the results with those of the Huggins, Miller, and Jensen method, I have accordingly determined the opacity per gram of protein and the iodoacetate index on 100 human sera, some from normal individuals and some from patients known to have malignant disease. The determinations of the iodoacetate index were carried out exactly as described by Huggins, Miller, and Jensen, except the proteins were found by the $CuSO_4$ density method instead of by micro-Kjeldahl. The coefficient of correlation between 100 observations of the opacity per gram of protein and the iodoacetate index was found to be 0.79 ± 0.038.[1]

RESULTS

Tests with small animals.—The results obtained for the opacity per gram of protein in normal and in tumor-bearing rats (Lewis sarcoma 304 and 311, benzpyrene tumors[2]) and in normal and in tumor-

[1] The results obtained with sera having an icterus index of 20 or more are not included. Sera with a high icterus index give unaccountably high opacities after heating, and the correspondence between the opacity per gram of protein and the iodoacetate index is poor. This situation is not improved by using red light or by subtracting the extinction coefficient for the diluted, unheated serum.

[2] Some of the tumor-bearing rats were obtained from the laboratories of the Rockefeller Institute, through the kindness of Dr. Keith R. Porter. Some of the tumor-bearing mice were obtained from the Roscoe B. Jackson Laboratory at Bar Harbor and some from Rockland Farms, N.Y.

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bearing mice (mammary tumors in swiss albino, dba line 1, and C3H strains; spontaneous lymphosarcomas) are shown in Table 1, together with values obtained in man (normal, and known to have malignant tumors of various types) which are given for comparison.

The distinct decrease in the opacity per gram of protein obtained in human sera from persons with various kinds of malignant tumors is also observed in the sera of tumor-bearing rats, but there is no indication of a similar difference between the sera of normal and tumor-bearing mice. The opacity per gram of protein is remarkably variable in the normal mouse.

Experience with the iodoacetate index and the opacity in human cancer.—In the course of the development of this method, considerable experience has accumulated about the significance of the iodoacetate index, as well as that of the reduced opacity of heated serum, in cases of cancer in man. False positives are about as frequent as Huggins, Miller, and Jensen report, and false negatives are more frequent than they indicate. These can occur, on repeated testing, in cases of metastatic carcinoma and even of carcinomatosis; the iodoacetate index and the opacity per gram of protein may accordingly be misleading as diagnostic tests. Another disturbing feature is the large number of "questionable" results, in which a difference between a "positive" and a "negative" test would result from coagulation occurring in adjacent tubes to the one selected. The end point of the coagulation test is none too sharp, and many clinically questionable cases fall into the questionable category from the standpoint of the test. Although the opacity per gram of protein is a figure which has no error attached to it, apart from that of the photometer reading and that of the protein determination, it is not possible to set up a value which will divide all normal human sera from all sera of persons with cancer. Again, the "questionable" class is too large.

TABLE 1

THE E VALUES PER GRAM OF PROTEIN IN NORMAL AND TUMOR-BEARING SUBJECTS

Man	Normal (50)	Mean = 0.121, $\sigma = \pm 0.018$
	Tumor-bearing (50)	Mean = 0.069, $\sigma = \pm 0.019$
Rat	Normal (20)	0.077 to 0.105, mean = 0.77
	Tumor-bearing (19)	0.026 to 0.080, mean = 0.39
Mouse	Normal (25)	0.022 to 0.170, mean = 0.33
	Tumor-bearing (14)	0.036 to 0.190, mean = 0.47

The disturbance in the coagulation mechanism and in the opacity-producing mechanism in cancer is nevertheless a real, if not a constant or a primary, phenomenon, and this method of measuring it is described, with an admittedly small number of animals, so that those who have colonies of tumor-bearing animals can make more extensive observations which readily suggest themselves and which can be made when the animals are killed for other purposes. Some of these questions are: in what kind of tumor-bearing animals does the phenomenon occur; at what time during the growth of a tumor does the phenomenon appear; and does it disappear when the tumor is removed?

SUMMARY

A method is described for measuring, as the extinction coefficient per gram of protein, the opacity developed by serum when heated under standard conditions. The values obtained are highly correlated with the iodoacetate index of Huggins, Miller, and Jensen, a decreased opacity corresponding to a decreased coagulability in cancer and other conditions in man. The quantities of serum required are so small that the method can be applied to small experimental animals; a similar decrease in opacity per gram of protein occurs in the tumor-bearing rat but has not been observed in the tumor-bearing mouse, largely because the opacity per gram of protein developed in heated mouse serum varies very widely.

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Studies on the Proteins of Rhabdomyosarcoma and Normal Muscle of Mice

I. Gross Composition, Extractions with 0.5 M KCl, and Fractionations by Differential Centrifugation and Dialysis

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INTRODUCTION

It has been pointed out by Toennies (28) that recent trends "from varied investigative directions, such as immunology, genetics and cytology, virus research and enzymology" have again focused attention on proteins and nucleoproteins as possible sites of specific chemical differences between malignant tumors and the normal tissues from which they arise. A survey of the earlier attempts to make comparative analyses of these components led to the conclusion that the difficulties encountered in the studies center around questions of "(*a*) homologous tissue, (*b*) homogeneous tissue, (*c*) deterioration of tissue, (*d*) disintegration of tissue, (*e*) the structural complexity of cells, and (*f*) the dynamics of metabolism." The present series of experiments represents the initiation of an attempt to overcome some of these difficulties and to obtain decisive evidence as to whether or not the proteins of cancer tissues differ in their chemical or physico-chemical properties from normal homologous tissues.

Normal, adult mouse muscle and a rhabdomyosarcoma derived from it were chosen as materials upon which a comparison might be based. The mice used were of an inbred strain (C3H) within which rhabdomyosarcomas were known to be readily producible (6). The tumors, once produced, could then be maintained by serial transplantation. Both the normal and malignant tissues were found to be relatively homogeneous, and a continuous supply of suitable homologous tissues was thus assured.

The use of muscle and muscle tumor had the further advantage that more information was available on the protein chemistry of muscle than of most other animal tissues. The proteins expected to be found in muscle include myosin (12, 27, 30), myogen (2, 4, 10, 24, 29), globulin x (16, 24, 31, 32), ribose and desoxyribose nucleoproteins (20), myoalbumin (24), muscle hemoglobin (19), actin (27), tropomyosin (3), peptomyosin (5), and numerous enzymes, some of which might be represented by certain of the above-mentioned proteins.

A preliminary analysis consisted of gross measurements of the moisture, ash, phosphorus, and nitrogen contents of whole tissues. This was followed by exploratory-extraction studies with a salt solution containing 0.45 M KCl and 0.05 M $KHCO_3$. A salt solution of such a concentration insures dissolving myosin and most of the other known proteins of muscle, with the exception of desoxyribose nucleoprotein. Preliminary fractionations of the extracts were carried out by differential centrifugation and by dialysis. The results of these studies are described in the present paper. An accompanying paper will report electrophoretic and viscometric studies of the extracts.

EXPERIMENTAL

Source and preparation of material.—In order to obtain a muscle tumor, 80 mice of the C3H strain, 2–2½ months old, were given a single injection of 0.5 mg. of 20-methylcholanthrene in 0.05 ml. of lard, filtered at 37° C. The mice were anaesthetized with nembutal, and the injection was made from the heel upward through the shank into the muscle of the thigh in 71 mice and directly into the muscle of the hip in nine mice. Of 69 mice that lived until the appearance of the first tumor, 57 developed tumors at the site of the injection, with in-

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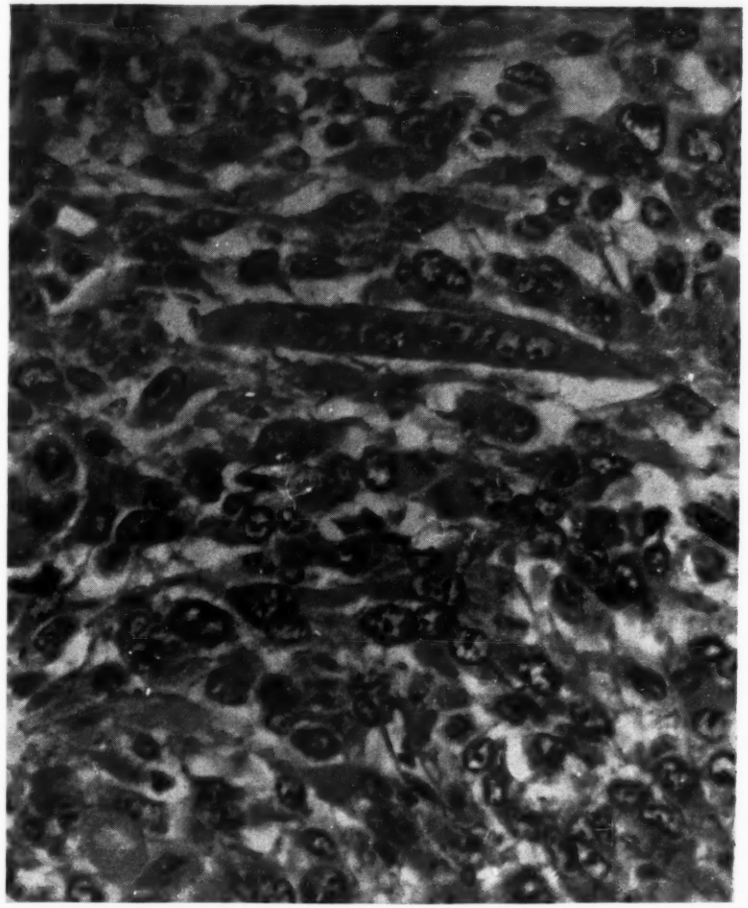

FIG. 1*a*.—Section of rhabdomyosarcoma (MC-1 A) induced in a C3H mouse, as it appeared in the first transplant generation. Zenker's with acetic. Galligher's hematoxylin, fast green.

FIG. 1*b*.—Section of normal mouse muscle. Bouin. Heidenhain's iron alum hematoxylin. Both × 1700.

duction times ranging from 71 to 323 days; and, of 52 tumors from which slides were available, 37 were spindle-cell or mixed spindle- and muscle-cell sarcomas, 7 were sarcomas of the polymorphous cell type, and 8 appeared to be pure rhabdomyosarcomas. Five tumors, representing all three types, were carried by serial transplantation.

The muscle tumor used in the present work (MC-1 A) was first observed 71 days after injection of methylcholanthrene. It was transplanted on the 95th day and was carried through more than 50 serial transplant generations. It was maintained in C3H and F_1 (C3H × dba) mice and was found to grow in 100 per cent of the inoculated animals. The rate of growth, however, was relatively slow, for, after 24 days, the tumors measured only about 1 × 1.5 cm. Figure 1 shows a section of the tumor as it appeared in the first transplant generation. For comparative purposes, a section of normal mouse muscle is also shown. The average composition per unit volume of typical sections of tumor tissue, as determined by the method of Chalkley (7), was 74 per cent tumor cells, 13 per cent necrotic tissue, and 13 per cent stroma and tissue space. Normal muscle contained 89 per cent muscle fibers plus 11 per cent of other tissues and tissue spaces. The ratio of volumes of cytoplasm to nucleus was 2:1 for the tumor cells and 50:1 for the normal muscle. The histological characteristics of the rhabdomyosarcoma did not change appreciably during successive transplantations. Longitudinal fibrils were distinguished in many of the smaller cells as well as in elongated giant cells, and occasionally cross striations were seen. Metastases occurred frequently in the lung and sometimes in other organs.

In order to prepare material for chemical studies, transplants were allowed to grow for 3–4 weeks in mice 3–5 months old. The mice were then killed by bleeding at the throat, and the tumors were excised. The weights of tissue obtained after removal of small amounts of necrotic material averaged 1.4 gm. per mouse. Normal muscle, when required, was obtained from either tumor-bearing or normal mice; the most readily dissected muscles of fore and hind limbs and back were used.

Deterioration of collected tissue was prevented by freezing it with dry ice. The material was either used at once or stored at −15° C. until needed. Before being analyzed directly or subjected to extraction, pieces of frozen tissue were mixed with approximately 2 volumes of dry ice and disintegrated to a fine powder, with the aid of a glass mortar and pestle. Suitable aliquots of the frozen powder were then transferred to containers in which they were next to be treated, and the weights were noted when all the dry ice had evaporated and when constant readings were obtained.

Gross composition of rhabdomyosarcoma and normal muscle.—Moisture determinations were carried out by drying 1-gm. samples of pulverized tissue *in vacuo* at 100° C. Constant weight was attained within 24 hours. For ash determinations, the dried residues were incinerated at 450° C. Total nitrogen determinations were made by a micro Kjeldahl-Nessler method (17). Phosphorus analyses were carried out by the method of Allen (1), and suitable aliquots of Kjeldahl digests were used for this purpose.

TABLE 1

GROSS COMPOSITION OF RHABDOMYOSARCOMA AND NORMAL MUSCLE

CONSTITUENT	NORMAL MUSCLE			RHABDOMYOSARCOMA	STD. ERROR OF DIFFERENCE OF MEANS	SIGNIFICANT DIFFERENCE
	Normal mice per cent	Tumor-bearing mice per cent	Mean per cent	per cent		
Moisture	75.3 (9)*	75.4 (7)	75.4 (16)	83.0 (11)	0.45	+
Ash, wet basis	1.264 (6)	1.326 (6)	1.295 (12)	1.302 (6)	0.093	−
Ash, dry basis	5.03 (6)	5.30 (6)	5.17 (12)	7.59 (6)	0.11	+
P, wet basis	0.249 (5)	0.242 (5)	0.246 (10)	0.262 (5)	0.006	−
P, dry basis	1.06 (5)	1.06 (5)	1.06 (10)	1.52 (5)	0.030	+
N, wet basis	3.49 (5)	3.42 (5)	3.46 (10)	2.23 (5)	0.046	+
N, dry basis	14.73 (5)	14.79 (5)	14.76 (10)	12.82 (5)	0.28	+

* The numbers of replicas are indicated in the parentheses.

Results obtained for rhabdomyosarcoma and for normal muscle from normal mice and mice bearing the transplanted tumor are shown in Table 1. Standard errors of means were calculated from the appropriate formula for small samples, given by Fisher (13). Significance of difference is based on a level of probability of 99 per cent. Since the data for normal muscle of normal mice did not appear to differ significantly from those of tumor-bearing mice, all data for normal muscle were averaged.

The moisture content of the cancer tissue was significantly higher than that of normal muscle. A high moisture content is a characteristic of cancer tissue, an observation encountered by many authors (25) but not yet explained. Furthermore, the actual values that were found seem to conform, in general, with those obtained by other

investigators on comparable materials. For example, the value for the normal muscle tissue of mice agrees well with the value previously reported for adult muscle of species other than mice (21, 25), while that for the tumor falls remarkably close to corresponding values reported for a number of tumors other than rhabdomyosarcoma (11, 14, 25). The high value for the muscle tumor of mice is also similar to the high values reported for bovine fetal muscle (21).

The ash contents differed when calculated on the basis of dry tissue but did not differ on a wet basis, and the phosphorus data showed analogous differences. The phosphorus of the tissues arose, presumably, from three major sources: namely, inorganic phosphate, adenyltriphosphate, and nucleoprotein. To judge on the basis of nuclear nucleoprotein alone, the 25-fold higher nucleocytoplasmic ratio of malignant tissue, compared with normal tissue, suggested an even greater difference in phosphorus values than was found. It seemed probable, however, that the adenyltriphosphate phosphorus of the tumor was lower than that of the muscle.

The nitrogen content of rhabdomyosarcoma was significantly lower than that of normal muscle, either on a wet or on a dry basis. The difference in nitrogen contents of normal and of tumor tissues might be due to differences in contents of fat, carbohydrate, or high or low nitrogen proteins. It might be noted that the respective values of 12.82 per cent and 14.76 per cent nitrogen for rhabdomyosarcoma and normal muscle of mice are quite similar to corresponding values of 11.81 per cent and 15.05 per cent nitrogen for fetal and adult bovine muscles (21).

The similarities of the high moisture and low nitrogen of mouse rhabdomyosarcoma to the high moisture and low nitrogen of embryonic muscle of the cow might be taken as expressions of the embryonic character of tumor. More reliable information on this question should be obtainable from a controlled comparison of embryonic and tumor tissues within the same species.

Extraction of tissues.—Differences in behavior of tumor and of normal tissues during extraction made it difficult to discover a single set of conditions suitable for both. During the course of the studies, therefore, several different conditions were used, as outlined under A, B, C, and D in Table 2. All extractions and centrifugations were carried out at 4° C. After the extractions were made with the buffered KCl, the residues which remained were extracted three times with 8 ml. of 0.1 N NaOH, leaving a small amount of fibrous material which resisted further dispersion. The various extracts were finally filtered by gravity through tight plugs of glass wool, to remove small amounts of suspended fat or other particulate material.

To follow the progress of the extractions, nitrogen determinations were carried out on each fraction. Calculations were then made of the distribution of nitrogen between the different extracts. It was found, however, that if corrections were applied to account for amounts of nitrogen assumed to exist in portions of fluid imbibed by the residues, anomalous results were obtained, particularly when the residues were well packed by high centrifugal fields. For example, less nitrogen was present in certain extracts than was assumed to occur in the imbibed fluids of the residues left from the previous extraction. This finding may be explained by the assumption that the imbibed fluid consists mainly of water of hydration. It would seem, therefore, that residues obtained after centrifugation at high speeds represent, principally, hydrated protein with comparatively little occluded protein solution. Consequently, the amount of nitrogen mobilized by any given extraction was calculated simply on the basis of the portion of fluid decanted from the centrifuged residue.

The first extraction experiments were carried out on tumor tissue under condition A. The distribution of nitrogen in per cent of total nitrogen is shown under experiments 1, 2, 3, and 4 of Table 3. On the average, 80 per cent of the nitrogen was extracted by the buffered KCl, 15 per cent by the alkali, and 5 per cent remained insoluble.

TABLE 2

CONDITIONS OF EXTRACTION WITH BUFFERED KCl

Conditions	A	B	C	D
Weight of sample (gm.)	8	8	8	8
Composition of extracting fluid	0.45 M KCl 0.05 M $KHCO_3$	0.45 M KCl 0.05 M $KHCO_3$ 0.001 M $Na_4P_2O_7$	0.45 M KCl 0.05 M $KHCO_3$ 0.001 M $Na_4P_2O_7$	0.45 M KCl 0.05 M $KHCO_3$ 0.001 M $Na_4P_2O_7$
Volume of extracting fluid (ml.)				
Extract No. 1	32	64	64	64
Extract No. 2	32	32	32	32
Extract No. 3	32	32	32	32
Extract No. 4	32	32	32	32
Time of shaking (min.)	30	30	30	30
Centrifugal field (*g*)*	2,000	2,000	8,000	8,000
Time of centrifugation (min.)	30	30	30	240

* Angle centrifuge, type SS-1, Ivan Sorvall, Inc.

When condition A was employed with normal muscle, however, stiff gels were obtained, and the extracts could not be used for further study. This difficulty was resolved by the use of condition C, by which the centrifugal field was made four times as great, the proportion of extraction medium used for the first extract was doubled, and 0.001 M $Na_4P_2O_7$ was incorporated into the medium to split the highly viscous actomyosin of muscle into the relatively less viscous actin and myosin (26). The distribution of nitrogen thus obtained is shown under experiments 5, 6, and 7 of Table 3. On the average, 68 per cent of the nitrogen was extracted by the buffered KCl, 26 per cent by the alkali, and 6 per cent remained insoluble.

To compare the rates of extraction of rhabdomyosarcoma and normal muscle under more nearly identical conditions, the extractions of rhabdomyosarcoma were next repeated under the conditions required by the muscle. Furthermore, to test separately the effects of pyrophosphate and high centrifugal fields, one experiment was carried out under condition B and another under condition C. The results, shown in Table 3, as experiments 8 and 9, respectively, indicate that the extraction data for the tumor tissue are not appreciably altered by either pyrophosphate or the higher centrifugal field. Comparison of the data for tumor with those for normal tissue shows that the nitrogen of the tumor is more readily and more completely extracted by buffered KCl than is the nitrogen of the normal tissue.

A further technical difficulty which contributed to the irregularities in certain data of Table 3 must be mentioned. This was caused by the occurrence of layers of fluffy material above the more firmly packed portions of the centrifuged residues. It became a matter of arbitrary choice to retain this material with the residue or to add it to the decanted extract. An unsuccessful attempt was made to solve the difficulty by the use of longer periods of high-speed centrifugation as in condition D shown in Table 2. This condition was completely unsatisfactory, however, for extraction of tumor tissue, since the residue obtained after the first extraction and centrifugation appeared in the form of a cohesive mass, completely unworkable for further extractions. It was concluded that short periods of high-speed centrifugation should be used during the successive extraction steps and that longer periods of centrifugation could then subsequently be carried out in order to remove fluffy material from the decanted extracts.

It was noted that, while the KCl extracts No. 1 of tumor were not at all viscous, all subsequent KCl extracts were very much so, even in spite of their lower nitrogen contents. Successive muscle extracts, on the other hand, showed viscosities which on superficial inspection corresponded roughly to their nitrogen contents.

Fractionation by differential centrifugation.—Preliminary observations were made in regard to the presence of "heavy" fractions in the various extracts. These were isolated by differential centrifugation. Extracts No. 1 of fresh muscle, obtained after ½-hour centrifugation at 8,000 *g* in experiment 5, were comparatively clear, and on further centrifugation for 3½ hours at 8,000 *g* yielded only very small pellets of heavy material. Extracts No. 1 of fresh tumor, as in experiment 9, on the other hand, were very opalescent after ½-hour centrifugation at 8,000 *g* and yielded large translucent pellets of heavy material on further centrifugation for 3½ hours at 8,000 *g*. Microscopic examination of the resuspended pellets revealed particles of a size roughly corresponding to Claude's "large granules" (8, 9). The yields of heavy material from an extract No. 1 of tumor after three successive centrifugations for 2½ hours each at 8,000 *g*. corresponded to 8.5, 3.3, and 1.8 per cent, respectively, of the total nitrogen of the extract. Suspensions of the material were not viscous.

Appreciable amounts of heavy material were also obtained from an extract No. 2 of tumor. The yields after two successive centrifugations for 2½ hours at 8,000 *g* corresponded to 21 per cent and

TABLE 3

DISTRIBUTION OF NITROGEN DURING EXTRACTION OF RHABDOMYOSARCOMA AND NORMAL MUSCLE

Exper. no.	Tissue	Conditions of extraction	KCl-$KHCO_3$ extractions					NaOH extractions	Insoluble residue
			No. 1	No. 2	No. 3	No. 4	Total		
			per cent						
1	Tumor	A	61.2	10.4	4.9	2.0	78.5	17.8	3.7
2	"	A	56.8	13.9	4.0	1.5	76.2	19.0	4.8
3	"	A	56.1	16.1	6.0	3.6	81.8	14.6	3.6
4	"	A	67.0	11.8	4.5	1.1	84.4	8.7	6.8
5	Normal	C	38.3	17.7	10.5		66.5	26.0	7.5
6	"	C	33.1	20.2	15.0	4.2	72.5	22.5	5.1
7	"	C	36.2	16.6	7.9	4.3	64.9	29.7	5.3
8	Tumor	B	63.9	15.1	2.7	1.7	83.4	14.0	2.5
9	"	C	55.7	19.9	4.0	1.8	81.4	16.0	2.6

4 per cent, respectively, of the total nitrogen of the extract. Suspensions of this material were highly viscous.

To distinguish heavy fractions of extract No. 1 of tumor from those of extract No. 2 by chemical means, tests were made for ribose nucleic acid and desoxyribose nucleic acid. These were carried out on trichloracetic extracts of the preparations (22) by modifications of the Mejbaum and Dische methods (18, 23). Heavy fractions from extracts No. 1 contained 10 per cent ribose nucleic acid and only 3 per cent desoxyribose nucleic acid, both values being calculated on the basis of an assumed nitrogen content of 11 per cent for the large granules (9). Heavy fractions from extracts No. 2 contained, on the other hand, 18 per cent desoxyribose nucleic acid, calculated on the basis of an assumed nitrogen content of 15 per cent for desoxyribose nucleoprotein (20). The ribose nucleic acid content of the latter material has not yet been determined.

The low viscosity and high ribose nucleic acid content of heavy material of extract No. 1 indicates it to be largely cytoplasmic granules, while the high viscosity and high desoxyribose nucleic acid content of heavy material of extract No. 2 indicates that this fraction is primarily nuclear nucleoprotein. It appears that the large granules, together with the bulk of the other cytoplasmic constituents, are taken up by the first extraction and that, following this step, subsequent extractions lead to incipient liberation of desoxyribose nucleoprotein from nuclear material remaining in the residues.

Fractionation by dialysis.—Since myosin is soluble in 0.5 M salt and insoluble in 0.02 M salt in which all other extracted proteins remain soluble (12, 24, 27, 30), it can be separated from the extracts by reduction of salt concentration. Dialysis of 20 ml. of extract No. 1 of normal muscle in $\frac{1}{4}$-inch cellulose casing against 480 ml. of distilled water at 0° C. for 2 hours with stirring, or overnight in the refrigerator, yielded a precipitate of myosin. The precipitate was separated by centrifugation and was freed of adhering supernatant fluid by resuspending it three times in 10 ml. of 0.02 M KCl and recentrifuging each time. The washed precipitate was then dissolved with the aid of 18 ml. of 0.5 M buffered KCl to yield a slightly opalescent, quite viscous solution. The yields of myosin isolated from extracts No. 1 of three different extraction experiments corresponded to 38–44 per cent of the total protein nitrogen of each extract.

The above dialysis conditions were next used for the isolation of myosin from tumor extracts. In each of the first two experiments, made on extracts No. 1 of stored tumor tissue, yields of myosin-like material represented 4.7 per cent of the total protein nitrogen of the extract. In the third experiment, carried out with extract No. 1 of fresh tumor tissue, a much higher yield of 13.7 per cent was realized. This was undoubtedly the result of contamination with "heavy" material which also was found to precipitate at low salt concentrations. In a fourth experiment, also carried out on fresh tumor tissue, precautions were taken to remove heavy material by means of repeated high-speed centrifugation before the dialysis step. From an extract thus treated, a final yield of myosin-like material of 5.3 per cent was obtained. To explain the lack of heavy material in the first two experiments, it must be assumed that storage of the tumor tissues in dry ice over a period of several months, as was the case, converted the heavy material into a form which did not separate with the myosin fraction.

Because of the possibility that the various myosin fractions might have been contaminated with nucleoproteins, tests were made for desoxyribose and ribose nucleic acid contents. The desoxyribose nucleic acid content of the tumor myosin fraction appeared very low, namely, about 2–4 per cent. The myosin fraction of normal muscle also gave a low value of 2 per cent or less. These values may justifiably be considered too high, since the color obtained was green, instead of blue, and the rate of color development was abnormally rapid. The ribose nucleic acid content of the myosin fraction from normal muscle was 1–2 per cent. Again, these low values which were obtained may not have been altogether due to ribose nucleic acid.

If it is assumed that the proportions of myosin in extracts Nos. 2, 3, and 4 were the same as those observed in extracts No. 1, the myosin content of rhabdomyosarcoma is about one-seventh that of normal muscle. This is similar to the low myosin content observed by Herrmann and Nicholas in embryonic muscle of the rat (15). In terms of general solubility, relative viscous behavior, and essential lack of nucleic acid, the myosin-like material from rhabdomyosarcoma is quite similar to myosin of muscle. It will be shown in the following paper, however, that the two differ in certain other respects.

SUMMARY

The production of a rhabdomyosarcoma in mice and its preparation for chemical analysis have been described.

Preliminary studies of gross composition of rhabdomyosarcoma and normal muscle revealed that the moisture content of rhabdomyosarcoma was significantly higher than that of normal muscle, while the reverse held true for the nitrogen

contents. The phosphorus and ash contents of tumor tissue were higher than those of normal tissues when calculated on the basis of dry tissue, but no differences were observed on a wet basis. The significance of these findings was discussed.

Extraction studies made with a salt solution containing 0.45 M KCl, 0.05 M $KHCO_3$, and 0.001 M $Na_4P_2O_7$ showed that the nitrogen of the tumor was more readily and completely extracted than that of the normal tissue. Other differences in behavior of the tissues during extraction were noted.

Fractionation of extracts by differential centrifugation yielded appreciable amounts of "heavy" material from tumor but very little from normal muscle. Tests for ribose nucleic acid and desoxyribose nucleic acid contents indicated that the heavy material obtained from a first extraction of tumor tissue represented largely cytoplasmic nucleoprotein, while heavy material from subsequent extracts represented primarily nuclear nucleoprotein.

Reduction of salt concentration of the extracts by means of dialysis, which yielded myosin from normal muscle, yielded also a myosin-like substance from tumor. The amount of this material obtained from tumor was approximately one-seventh that obtained from muscle.

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Studies on the Proteins of Rhabdomyosarcoma and Normal Muscle of Mice. II. Electrophoretic and Viscometric Measurements

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INTRODUCTION

Proteins are ordinarily characterized, from a physico-chemical standpoint, in terms of their electrophoretic behaviors and the sizes and shapes of their molecules. In order to compare proteins of cancer tissues with those of normal tissues it is desirable, therefore, to establish these properties for each. In addition, it is of interest to determine the relative proportions in which the different proteins occur. A feasible approach consists of extracting tissues with appropriate solvents and subjecting the fractions obtained to electrophoresis, ultracentrifugation, diffusion, partial specific volume determination, and viscometry. The extraction of rhabdomyosarcoma and normal muscle of mice with buffered 0.5 M salt solution and the partial fractionation of the extracts by differential centrifugation and dialysis have been described in the preceding paper (16). The present work covers exploratory electrophoretic and viscometric studies of these extracts and the fractions obtained therefrom. Results obtained with other extraction solvents and by other physico-chemical methods will serve as bases for subsequent reports.

EXPERIMENTAL

Electrophoretic measurements.—Electrophoretic measurements were made in the Longsworth modification (12, 13) of the Tiselius apparatus, purchased from the Klett Manufacturing Company. Observations of the boundaries were made with the Svensson-Philpot optical system (19). Since most of the extracts were opalescent, photographs of the boundaries were taken with the aid of infrared light and infrared sensitive plates, as suggested by Treffers and Moore (21). Fifteen-ml. aliquots of the samples to be used for electrophoresis were placed in cellophane tubes and equilibrated for 48 hours against 2-l. portions of buffer.

Most of the electrophoresis experiments were carried out in 0.45 M KCl-0.05 M $KHCO_3$ containing 0.001 M $Na_4P_2O_7$. This was the same buffer which was used for the tissue extractions (16), and its further use for electrophoresis avoided the precipitation of proteins which might occur during dialysis against buffers of other ionic strengths or pH values. It had been noted during the previous studies that the presence of 0.001 M $Na_4P_2O_7$ was essential to the extraction of normal muscle but did not affect appreciably the extraction of rhabdomyosarcoma (16). In complete analogy, the presence of pyrophosphate was essential to the electrophoresis of extracts of normal muscle but did not affect the electrophoresis of extracts of tumor tissue. It must be pointed out, however, that although electrophoresis was thus practicable on all successive extracts of muscle, only the first extract of tumor could be studied. Extracts Nos. 2, 3, and 4 of tumor were much too viscous, and, further, this condition was not alleviated by the addition of pyrophosphate.

After the removal of the myosin from the extracts by the procedure previously described (16) it was found that dialysis against the 0.1 N veronal buffer described by Longsworth (14) could be carried out without any precipitation of other constituents of the extracts. For comparative purposes, electrophoretic measurements were, therefore, made in this buffer, as well as in the KCl buffer, on the supernatants from the precipitates of the myosin fractions.

When the 0.5 M buffered KCl was used, it was not possible to employ a current greater than 30 milliamperes, and this necessitated running the experiments for at least 24 hours. The danger of polarization of the electrodes during these long experiments was avoided either by stopping the current and exchanging the electrodes or by starting the experiments with electrodes of which one had been completely coated with silver and the other coated with silver chloride. By using these

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"charged" electrodes, an experiment could be carried out for as long as 38 hours without polarization. When the 0.1 N veronal buffer was used, however, these difficulties were not encountered, and it was possible with a current of 20 ma. to complete the runs within 3–4 hours.

The "δ boundary anomaly" (15) appeared in runs which were made in the 0.1 N veronal buffer but not in those made in the 0.5 M buffered KCl. With both buffers the patterns obtained from ascending and descending boundaries were usually very similar in appearance. The mobilities calculated for corresponding boundaries of ascending and descending limbs were averaged.

Representative electrophoretic patterns obtained for extract No. 1 of rhabdomyosarcoma and extract No. 1 of normal muscle are shown in Figure 1, *a* and *b*, respectively. Electrophoretic diagrams for extracts of normal muscle succeeding extract No. 1 are not shown, since they were very similar to those of the first extract. The patterns for rhabdomyosarcoma revealed four major components: those for normal muscle, three major components, plus several very minor ones. The minor components will not be treated in this report. The mobilities of the major rhabdomyosarcoma components, arbitrarily labeled α, β, γ, and δ were 0.28, 0.35, 0.56, and 1.01×10^{-4} cm/sec/v/cm, respectively; those of normal muscle, labeled A, B, and C, were 0.24, 0.28, and 0.35×10^{-4} cm/sec/v/cm. These values represented averages obtained from extracts No. 1 of seven different preparations of rhabdomyosarcoma and extracts Nos. 1, 2, and 3 of three different preparations of muscle.

It can be seen that the δ component of rhabdomyosarcoma has no counterpart in the muscle. Because of its high mobility, it is thought likely to represent nucleic acid. Components α and β of tumor appear at first glance to resemble B and C of muscle; later experiments, to be described, prove, however, that they are different. The α and β components of rhabdomyosarcoma and the A and B components of muscle were usually very poorly resolved. A possible component in tumor extracts migrating at a rate between those of the β and γ components was not usually distinguishable.

The proportions in which the different components of the extracts occurred were calculated on the basis of the areas under the individual peaks. This was not possible for all components, due to their incomplete electrophoretic separation. Thus, the α and β of tumor had to be considered together and similarly the A and B of muscle. Components α plus β comprised 87 per cent of the total proteins of extract No. 1 of tumor; γ and δ, 9 and 4 per cent, respectively. Components A plus B comprised 42 per cent of the total proteins of extract No. 1 of normal muesle; C, 58 per cent.

Myosin preparations, obtained from extracts of tumor and normal tissues (16) revealed electrophoretic patterns such as those presented in Figure 1, *c* and *d*, respectively. Boundaries of the myosins were often somewhat asymmetric owing to the viscous nature of the proteins, as pointed out by Dubuisson (4). The mobility of tumor myosin, averaged from the values given by three different preparations, was 0.38; that of muscle myosin, also averaged from those of three different preparations, was 0.33. The mobility of the myosin of normal muscle corresponded to that of the C component of the original muscle extract.

Supernatants from the myosin preparations, containing the proteins which were soluble in 0.02 M salt (16), exhibited the electrophoretic diagrams shown in Figure 1, *e* and *f*. The diagrams of the supernatants of tumor extracts showed no significant change from the patterns of the original tumor extracts; the slight variations were of the same type and magnitude as those given by different preparations of the original extracts. This was to be expected, however, since the amounts of myosin removed from tumor were too low to be revealed in the presence of large amounts of other components having approximately the same mobility. Diagrams for the supernatants from normal muscle myosin showed, on the other hand, a marked alteration, because they lacked the C component. It may be concluded, therefore, that the C component of extracts of normal muscle represents myosin.

In order to obtain more detailed information regarding the proteins of the supernatants, it appeared worth-while to carry out electrophoretic tests in 0.1 N veronal buffer at pH 8.6. As mentioned earlier, the lower ionic strength could be employed once the myosin was removed. Furthermore, the lower ionic strength made it possible to obtain more rapid movement of the boundaries, together with improved resolution of components by virtue of minimized spread due to diffusion. Diagrams of the rhabdomyosarcoma and muscle supernatants, obtained under these conditions, are shown in Figure 2, *a* and *b*, respectively. Those of the tumor material revealed with fair certainty a total of seven components. In the diagram shown, the most rapidly moving one, the δ component, has passed out of the field. These components were labeled α_1, α_2, α_3, β_1, β_2, γ, and δ, and were found, in four experiments, to yield average mobilities of 0.21, 0.32, 0.39, 0.47, 0.57, 0.68, and 0.98, respec-

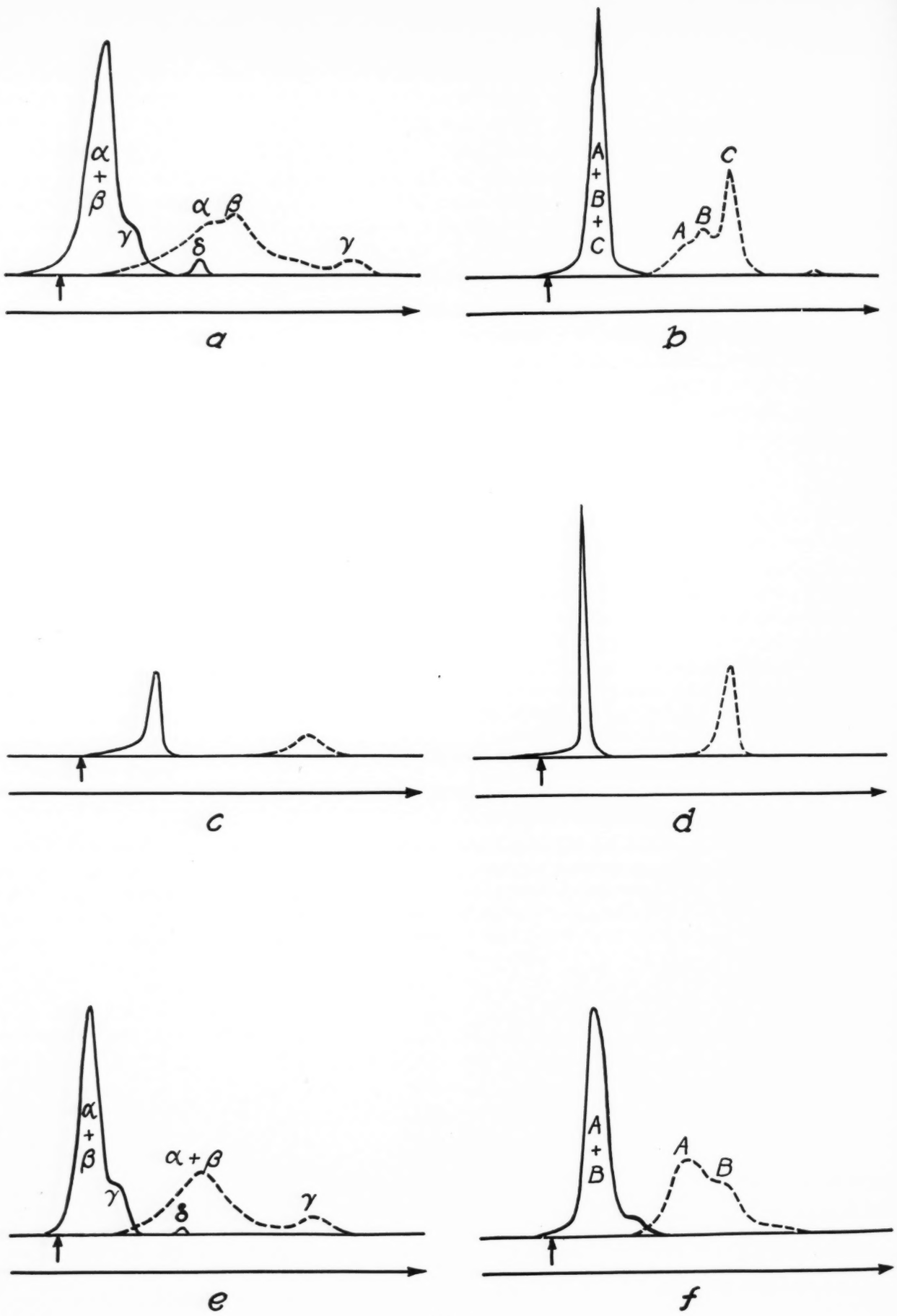

FIG. 1

tively. Diagrams of the muscle supernatants revealed improved resolution still more markedly than did the tumor supernatants, but no new components appeared. Values for the mobilities of A and B, averaged from two experiments, were 0.21 and 0.35. From the results as a whole, obtained with veronal buffer, it may be observed that only the mobilities of the a_1 component of tumor and the A component of muscle were the same, but further evidence would be required to prove their identity.

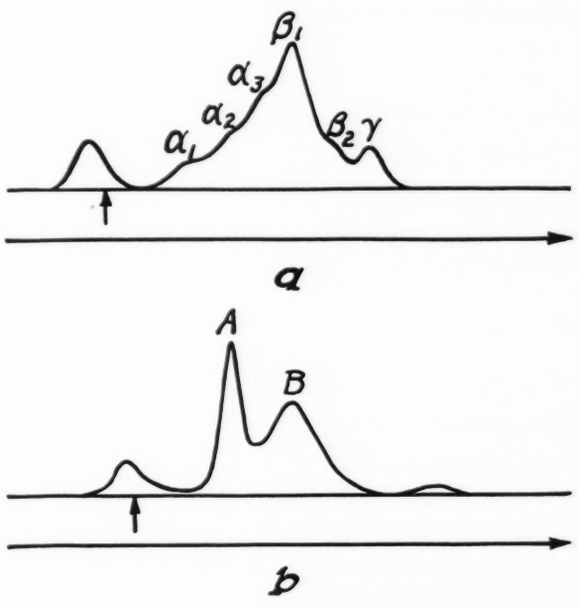

FIG. 2.—Electrophoretic patterns of ascending boundaries of fractions of rhabdomyosarcoma and normal muscle of mice. Buffer, 0.1 N veronal at pH 8.6. The vertical arrows indicate positions of starting boundaries; the horizontal arrows, directions of migration. Time of electrophoresis, 2 hours at a field strength of 8.1 v. per centimeter. (*a*) Supernatant from myosin of extract of rhabdomyosarcoma. Protein, not determined. Angle, 70°. (*b*) Supernatant from myosin of extract of normal muscle. Protein, not determined. Angle, 70°.

The pentose-containing heavy fractions obtained from extract No. 1 of tumor (16) proved to be remarkably homogeneous when tested by electrophoresis. Only a small proportion of the material, possessing a much higher mobility, separated from the principal component. Patterns given by the heavy material, tested in buffered 0.5 M KCl, are shown in Figure 3. The mobility of the major component, averaged from two experiments, was 0.36.

Because of several sources of error which were encountered in the long experiments carried out in buffered 0.5 M KCl, such as the increased opportunity for leakage, the boundary spread due to diffusion, and the variable pH due to the poor buffering power of the $KHCO_3$, values for mobilities determined under these conditions could not be considered to be of high accuracy. In order to establish with certainty the identity or nonidentity of the particular proteins whose mobilities determined in the buffered KCl fell within the same range, it was necessary, therefore, to carry out special electrophoretic runs on mixtures of such fractions. A mixture of myosin from tumor and myosin from normal muscle, thus tested, gave two distinct boundaries, having mobilities of 0.45 and 0.33, respectively. It was thereby well established that the myosins from the two sources differ electrophoretically, even though other properties would appear to place them in the same classification (16). The abnormally high mobility of the

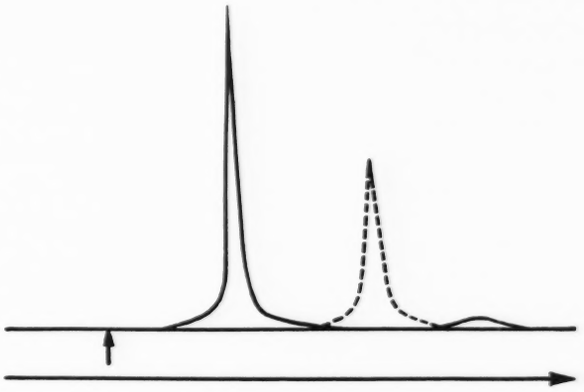

FIG. 3.—Electrophoretic patterns of ascending boundaries of ribose-containing heavy fraction from extract of rhabdomyosarcoma. Buffer, 0.45 M KCl–0.05 M $KHCO_3$–0.001 M $Na_4P_2O_7$. The vertical arrows indicate positions of starting boundaries; the horizontal arrows, directions of migration. Solid line represents the boundaries after approximately 12 hours at a field strength of 1.3 v. per centimeter; broken lines, after approximately 24 hours. Protein, 4.7 mg. per milliliter. Angle, 60°.

FIG. 1.—Electrophoretic patterns of ascending boundaries of fractions of rhabdomyosarcoma and normal muscle of mice. Buffer, 0.45 M KCl–0.05 M $KHCO_3$–0.001 M $Na_4P_2O_7$. The vertical arrows indicate positions of starting boundaries; the horizontal arrows, directions of migration. Solid lines represent boundaries after approximately 6 hours at a field strength of 1.3 volts per centimeter; broken lines, after approximately 24 hours. (*a*) Extract of rhabdomyosarcoma. Protein, 7.0 mg. per milliliter. Angle of knife edge, 70°. (*b*) Extract of normal muscle. Protein, 10.3 mg. per milliliter. Angle, 50°. (*c*) Myosin from extract of rhabdomyosarcoma. Protein, 2.9 mg. per milliliter. Angle, 70°. (*d*) Myosin from extract of normal muscle. Protein, 3.6 mg. per milliliter. Angle, 50°. (*e*) Supernatant from myosin of extract of rhabdomyosarcoma. Protein, 6.0 mg. per milliliter. Angle, 70°. (*f*) Supernatant from myosin of extract of normal muscle. Protein, 5.6 mg. per milliliter. Angle, 70°.

tumor myosin in the presence of normal myosin will, however, require further investigation.

A mixture of heavy material of tumor with myosin of tumor yielded on electrophoresis a slightly asymmetric peak, with a mobility of 0.37. This finding was in accord with the observations already made that the mobilities of each of these substances determined separately were quite close to one another.

Viscometric measurements.—Viscometric measurements were made in an Ostwald viscometer of 1 ml. capacity. Aliquots of extracts and fractions which had been equilibrated against buffers for purposes of electrophoresis were used also for viscometric measurements. The concentrations of protein in the test samples were 1–6 mg. per ml., presumably low enough to avoid the anomalous viscosities which are more likely to appear at high concentrations. By carrying out the measurements at 1.8° C. and in media which always contained 0.001 M $Na_4P_2O_7$, the dissociation of extremely viscous actomyosin, which might be formed in certain extracts, was assured (18). It was found convenient to make the determinations in the constant temperature bath of the electrophoresis apparatus and to observe the meniscuses from the electrophoresis camera.

The viscosity data were first calculated in terms of specific viscosities, as defined elsewhere (11). Then, by use of the factor 0.25 per milligram per milliliter, which is the specific viscosity of rabbit myosin per unit concentration (8), it was possible to estimate the amounts of myosin present in different extracts. It was assumed for these calculations that the viscosity of myosin of mouse tissues would not differ appreciably from that of myosin of rabbit tissues.

The efficacy of pyrophosphate in minimizing the viscosities of muscle extracts was established quantitatively by the following viscometric studies. First, an extract of muscle was prepared with buffered KCl containing no pyrophosphate. After the extract had stood for 2 days at 4° C., it was tested in the viscometer. Its specific viscosity indicated that 63 per cent of the protein nitrogen was represented by myosin. When pyrophosphate was added to an aliquot of the extract, a slightly lower viscosity resulted, corresponding to a myosin proportion of 56 per cent. Three days later the tests were repeated. It was then observed that the original extract had become much more viscous and appeared to contain 131 per cent myosin; but, after treatment with pyrophosphate, a lowered viscosity was obtained, which again corresponded to only 55 per cent. The results of this experiment were confirmed repeatedly. The same findings were observed whether the concentration of pyrophosphate was 0.001 M or 0.01 M. Extracts of three other muscle preparations showed, in the presence of pyrophosphate, viscosities which corresponded to 57, 61, and 68 per cent myosin, respectively. Szent-Györgyi and his associates (1, 2, 17, 20) have explained the low viscosity of fresh muscle extracts compared to that of stored extracts as due to adenyltriphosphate in the former, which promotes the dissociation of highly viscous actomyosin into the less viscous actin and myosin. Over a period of time, the viscosity increases due to the decomposition and loss of the adenyltriphosphate. The reversibility of the change may then be demonstrated by the addition of adenyltriphosphate, or of pyrophosphate, as shown by Straub (18).

In contrast to the behavior of muscle extracts, tumor extracts were found to show no change in viscosity on standing or on addition of pyrophosphate. This suggests a reduced quantity of actin in extracts of tumor. The value for the specific viscosity of tumor extracts corresponded to a proportion of only 17 per cent myosin.

The above values for apparent myosin contents of normal muscle and of tumor represent upper limits, since they are based on an assumption only partially justified, namely, that myosin is the only viscous substance present in the extracts. The presence of appreciable amounts of desoxyribose nucleoprotein, for example, is not altogether excluded, and, in fact, is particularly likely in the tumor extracts (16).

Preparations of muscle myosin were found to possess specific viscosities of 0.24–0.33 per milligram per milliliter, as obtained for four different samples. Two other preparations possessed extremely high viscosities, so that further studies will be required to establish the best conditions for obtaining preparations of myosin of minimal viscosity. Since denatured myosin would be likely to possess an abnormally high viscosity, it may be assumed that the myosin preparations of lowest viscosities represent material in the most nearly native state. The lowest viscosity obtained, namely, 0.24, agrees well with the value of 0.25 reported for myosin obtained from rabbit muscle (8).

Tumor myosin was found to possess a specific viscosity of only 0.133–0.144 per milligram per milliliter, as observed for two different preparations tested. These values are significantly lower than those of normal muscle myosin and suggest that molecules of tumor myosin are less asymmetric than those of normal muscle.

Preparations of heavy material from tumor possessed specific viscosities of 0.042–0.043 per milligram per milliliter, as shown by two samples

tested. Particles of heavy material appear, therefore, to be less asymmetric than molecules of either muscle or tumor myosins.

Supernatants from myosin of normal muscle showed specific viscosities of only 0.01–0.02 per milligram per milliliter. When the specific viscosities were calculated in terms of amounts of myosin present, it was found that not more than 4–8 per cent of the total myosin of the original extracts remained in the supernatants. Supernatants from myosin of tumor extracts were not studied in the viscometer.

DISCUSSION

The results of the present study confirm the well-recognized importance of using several tests in determining the identity or nonidentity of different proteins. For example, the over-all solubility characteristics of the myosins of tumor and of muscle suggested their possible exact identity, yet their differing electrophoretic mobilities and specific viscosities demonstrate that they are not identical. In another case, the similar mobilities of the myosin from tumor and the heavy fraction from tumor might have pointed toward their identity, were it not that their contents of ribose nucleic acid were totally different (16) and that their specific viscosities also differed markedly. Finally, the mobilities of the α and β components of the tumor appeared the same as those of the B and C components, respectively, of normal muscle when the tests were made in 0.5 M buffered KCl; but, first, the nonidentity of the β and C components was proved by the fact that only the latter was found to represent myosin; and, secondly, when electrophoretic tests were made in 0.1 N veronal buffer, the α component of tumor was found to represent a composite of at least three species, only the slowest of which, the α_1 component, agreed in mobility with one of the muscle components, namely, the A component.

It appears that no certain evidence has yet been obtained for the identity of any of the proteins of rhabdomyosarcoma with those of normal muscle. There are several reasons which might account for such a finding. In the first place, previous investigators have isolated from normal muscle by chemical procedures many more components than have been detected electrophoretically in the present study. Some of these substances may be present in such small amounts that they do not show up electrophoretically, when included in a common extract. For example, it is known that certain enzymes may be lacking in tumors or may be present in such reduced amounts that they would escape electrophoretic detection (7). It is also probable that, in some instances, what have been referred to as major electrophoretic components actually represent groups of substances having the same or nearly the same mobilities, as was initially the case with myosin and the "heavy" material of the rhabdomyosarcoma. On this basis, the possibility of the identity of some of the components in muscle and tumor has not been excluded.

Secondly, an apparent lack of identify of certain homologous components of the tumor and of normal adult muscle may be accounted for by differences in the degree of differentiation of the tissues. It has already been pointed out (16) that the cells of the tumor differ from those of adult muscle in their nucleo-cytoplasmic ratio and in the lesser and actually varying degree of differentiation of the cytoplasm. It is not inconceivable that the three α components found in the tumor extracts represent different forms of a single protein, which could correspond to the A component of normal muscle, although further analysis would be necessary to show whether or not this is the case. The myosin fractions are another case in point. The relatively low quantity of myosin-like substance in the tumor is comparable to the low myosin content found by Herrmann and Nicholas in embryonic muscle in the rat (9) and analogous to the low yield of collagen obtained from fibrosarcoma, as compared with differentiated connective tissue in the rat (5). While, in the present study, the myosins from the tumor and normal muscle were similar in general solubilities, they were found to differ (*a*) in their electrophoretic mobilities and (*b*) in their specific viscosities. Whether these differences will be found to correspond to the characteristics of the myosin sub-fractions obtained by Herrmann and Nicholas, which were found to change in the proportion of their occurrence during embryonic development, will remain to be seen.

The presence of a more conspicuous quantity of "heavy material" in the tumor than in the adult muscle is of interest. The relatively large amount of desoxyribosenucleoprotein obtained in second and subsequent extracts of the rhabodmyosarcoma reflect the high nucleo-cytoplasmic ratio of the tumor. As to the ribose-containing material separated after the first extraction, it should be pointed out that, with the speed of centrifugation used here (8,000 *g*), this comprises mainly the large cytoplasmic granules, while the microsomes, in all probability, remain in the supernatant fluid. Analysis with the ultracentrifuge will reveal whether or not this is the case.

The comparison of electrophoretic patterns of crude extracts and myosin fractions of mouse muscle, described in the present paper, with corresponding patterns obtained by other investigators

for preparations of muscle of other species (3, 6, 10) merits a separate study.

SUMMARY

Exploratory electrophoretic and viscometric studies have been made of extracts of rhabdomyosarcoma and normal muscle of the mouse and of certain fractions obtained therefrom.

Electrophoretic measurements revealed seven major components in the tumor extracts and three major components in the muscle extracts. Mobility data indicated that none, except possibly one, of the major components of tumor is identical with any of those of normal muscle. Myosin preparations isolated from each type of tissue also differed in their mobilities. The possibility that some of the major components revealed by electrophoresis may represent groups of components was pointed out, and its significance was discussed.

The ribose-containing heavy material obtained from tumor tissue by centrifugation at 8,000 *g* was studied electrophoretically and found to be quite homogeneous.

Viscometric studies of the myosin of tumor and of muscle revealed that the former was significantly less viscous than the latter. The ribose-containing heavy material was found to be still less viscous. These results were interpreted to indicate that molecules of tumor myosin were less asymmetric than those of normal muscle myosin, while particles of heavy material were still less asymmetric.

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The Distribution of Radioactive Iodine in Mice with and without Tumor 15091a after Injection of Radioactive Sodium Iodide*

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In studying the distribution of radioactivity in animals after administration of organic compounds labeled with radioactive iodine (1, 5, 7), it is useful to know for comparison the distribution of administered inorganic iodide. This distribution is also of interest in the study of iodine metabolism. Investigations reviewed by Lerman (2) and others (6) indicate that the thyroid gland is affected by extra-thyroid cancers as shown by measurement of the metabolic rate in man and by histological examination of the thyroid in animals. That the metabolism of I^{131} is altered in mice with cancer is also suggested by recent work with labeled thyroglobulin (4). We found that the distribution of administered iodide labeled with I^{131} in rats was altered by the presence of Walker tumor 256 (8). Somewhat different alterations found in mice with the mammary carcinoma, 15091a, are described in this report.

EXPERIMENTAL

The analytical methods used were similar to those described previously (8). To indicate the reliability of these methods in the present work, a mouse was given by tail vein 37.9 μg. of iodine as sodium iodide labeled with 6.4 μc.[1] of I^{131} and was killed 15 minutes later. A total of 93 per cent of the injected iodine was accounted for by measurement of the radioactivities of the nineteen parts into which the animal was divided.

Three principal experiments were carried out, using, altogether, 206 mice. While in this laboratory they were fed Purina Fox Chow and tap water *ad lib.* In the first experiment, labeled iodide was given intravenously to 56 young male ABC mice; of these, tumor 15091a[2] had been implanted into 28, 11–16 days previously. The control and tumor mice were grouped and paired according to size and were injected alternately. The mice were killed by severing their neck vessels, either 45 minutes or 25 hours after injection. The tissues were weighed immediately, and their radioactivities were measured as before (8). The thyroids were not dissected from the tracheas, but that portion of each trachea immediately below the larynx was removed with the attached thyroid, minced, and air-dried in the center of a cover glass. One-tenth-ml. aliquots of blood were pipetted directly onto cover glasses. Sufficient counts were taken with most aliquots to reduce the standard errors of the counting rates below ± 7 per cent.

The distribution of the injected I^{131} differed significantly between the control and the tumor mice for both their thyroids and their spleens but not for the other tissues examined (Table 1). These differences were found both in mice killed at 45 minutes and in those killed at 25 hours after iodide injection, and both in mice which received the large quantity and in those which received the small quantity of iodide. The thyroids of the tumor mice contained less I^{131} than did those of the controls. The spleens of the tumor mice, in all but four instances, contained higher concentrations of I^{131} than did those of their controls—generally higher than the concentrations present in the blood. The tumor mice had enlarged spleens, but no consistent relation was observed among the individual mice between the degree of enlargement and the concentration of I^{131} in the spleens. The livers and kidneys of the tumor mice were also enlarged,

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[1] All absolute values for radioactivity are based upon information furnished by the Clinton Laboratories and not upon measurements made in this laboratory.

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[2] Obtained from the Roscoe B. Jackson Memorial Laboratory.

TABLE 1

THE ORGAN WEIGHTS AND THE CONCENTRATIONS OF INJECTED IODINE IN THE TISSUES OF 27 ABC MICE, 13 EACH WITH TWO SUBCUTANEOUS IMPLANTS OF TUMOR 15091a AND 14 CONTROLS

These mice were killed 45 minutes after intravenous injection of 0.015 cc. of sodium iodide solution labeled with 0.4 μc. of I^{131} per gram of body weight. The values given are averages, except those for the spleens which are given individually, descending in the column in order of increasing body weight of the paired tumor and control mice.

Tissues	Mice with tumor		Control		Mice with tumor		Control	
	Body weight at the time of the injections							
	17.0 gm.		15.9 gm.		15.8 gm.		15.5 gm.	
	Amount of iodine injected per gram of body weight							
	1.45 mμg		1.39 mμg		1.24 μg		1.22 μg	
	Injected iodine per gm. (mμg)	Fresh wt. of organs (mg.)	Injected iodine per gm. (mμg)	Fresh wt. of organs (mg.)	Injected iodine per gm. (μg)	Fresh wt. of organs (mg.)	Injected iodine per gm. (μg)	Fresh wt. of organs (mg.)
Blood	2.3		2.1		1.10		1.39	
Spleen	4.8	260	1.8	59	3.1	138	0.87	54
	10.7	85	1.7	38	4.4	98	0.60	40
	15.2	76	1.5	70	2.9	173	0.45	79
	4.0	166	1.0	42	5.2	171	1.0	47
	4.2	142	1.5	42	4.5	175	4.6	39
	4.0	230	10.0	87	3.3	122	0.94	75
	1.7	290	1.5	46			1.4	38
Liver	0.76	850	0.77	960	0.71	810	0.57	970
Kidneys	1.12	250	1.09	230	0.97	230	0.88	215
Lungs	1.8	106	1.5	104	1.2	115	1.0	112
Heart	1.2	76	0.74	66	0.84	58	1.1	56
Leg muscle	0.40		0.43		0.33		0.32	
Stomach	7.0	69	5.1	58	4.5	63	5.5	52
Forestomach	1.7	27	2.9	24	1.7	23	1.6	23
Testes	0.65	105	0.58	125	0.60	83	0.53	134
Small tumor	1.16	1,250			0.91	1,210		
Large tumor	0.94	1,950			0.84	1,500		
Thyroid (total per gland)	0.22		0:46		0.02		0.07	

TABLE 2

THE AVERAGE CONCENTRATIONS OF INJECTED IODINE IN TISSUES

60 CFW male mice killed 45 minutes after intravenous injection of 0.014 cc. of sodium iodide solution labeled with 0.6 to 0.05 μc of I^{131}, per gram of body weight. The column of average values includes data obtained on the 25 mice (three groups) which received 0.1 cc. instead of the 0.5 cc. of vaccine or phenol injected into the mice in the three groups listed below. One mouse from each of the seven groups was killed at either 4, 7, 9, 12, 14, 16, 17 or 18 days, respectively, after injection of the vaccine or phenol.

	Materials injected intravenously 4–18 days earlier				
	Triple typhoid vaccine	Typhoid combined vaccine	0.45 per cent phenol	Not injected	Average values
Number of mice	9	9	7	10	60 (total)
Average body weight, gm.	22.4	22.9	21.8	24.0	22.4
Spleen weight in per cent of body weight	1.16	1.40	1.28	1.25	1.21
mμg of added iodine injected per gram of body weight	1.37	1.31	1.35	1.34	1.34
Tissues	mμg of Added Iodine Found per Gram of Fresh Tissue				
Serum	2.01	1.78	1.97	2.51	2.02
Spleen	0.76	0.73	0.74	0.70	0.75
Liver	0.63	0.63	0.57	0.65	0.66
Kidneys	1.20	1.29	1.35	1.39	1.30
Lungs	1.26	1.15	1.11	1.28	1.20
Heart	0.54	0.52	0.56	0.59	0.54
Leg muscle	0.36	0.26	0.27	0.33	0.31
Stomach	6.7	7.2	5.6	7.3	6.6
Forestomach	2.3	2.1	1.8	2.8	2.3
Testes	0.63	0.47	0.52	0.51	0.50
Ear pinna	0.96	0.86	0.94	0.98	0.93
Thyroid (total per gland)	0.60	0.77	0.80	0.81	0.70

though less markedly. At 25 hours after injection of iodide, the concentrations of I^{131} in the tissues other than the thyroid were 5–10 per cent of those present at 45 minutes after injection.

A second experiment was planned to learn if the production of splenic enlargement in some other way would result in altered distribution of injected 0.45 per cent phenol; one group was left uninjected. Four to 18 days later they were injected with labeled iodide, and 45 minutes later they were killed by bleeding from the heart during ether anesthesia. In this and in later experiments, serum was used instead of blood. In measuring radioactivity, sufficient counts were taken with most of

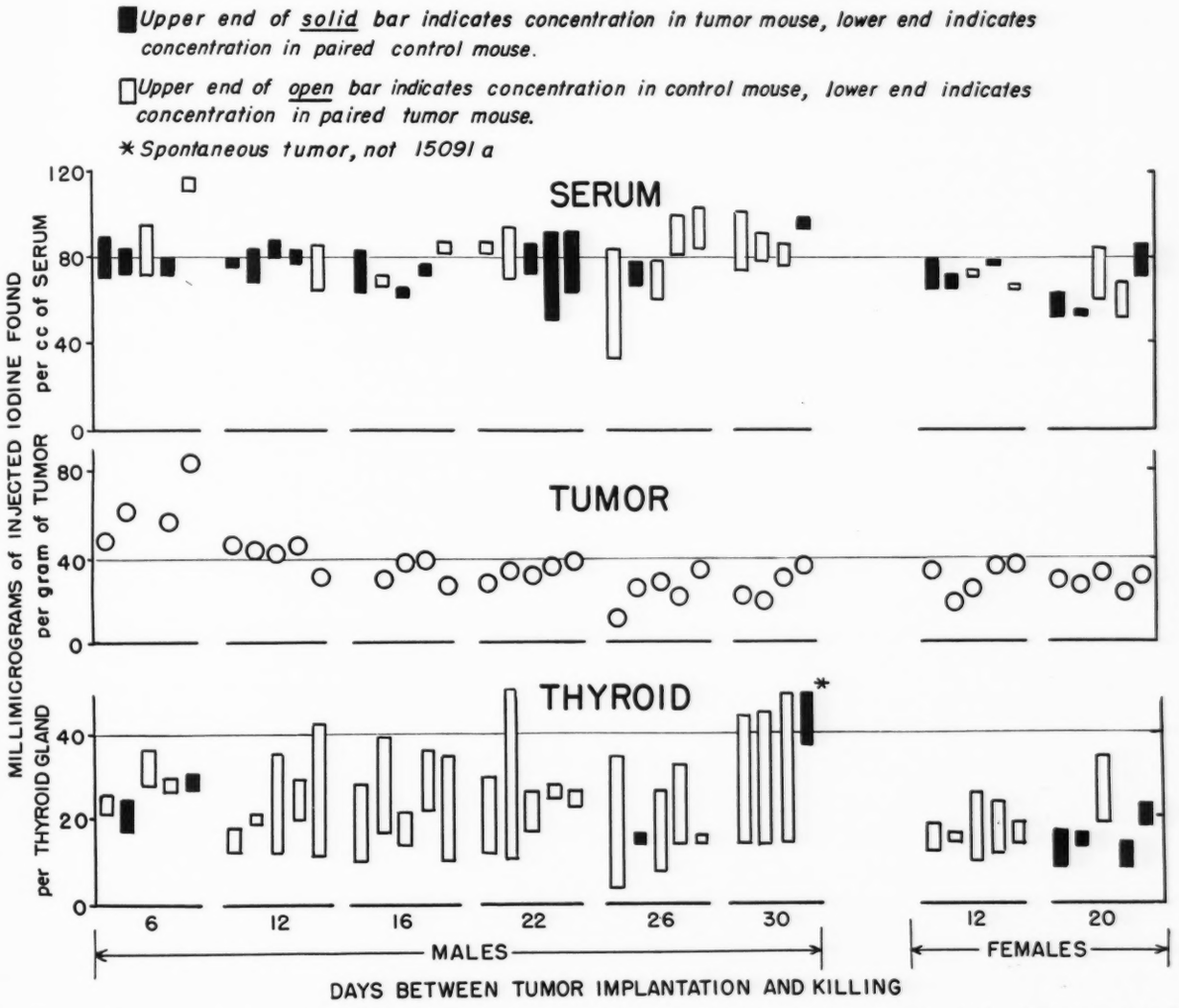

FIG. 1.—The concentrations of injected iodine found in the sera and tumors and the total quantities of injected iodine found in the thyroid glands of the 58 male and 20 female ABC mice in the third experiment. Each mouse received 48 mμg. of added inert iodine in 0.011 cc. of solution labeled with 0.25 to 0.11 μc. of I^{131} per gram of body weight and was killed 45 minutes later.

iodine. At this time the supply of ABC mice was cut off by the fire at the Jackson Memorial Laboratory. Sixty mice of the CFW strain were therefore used. They were divided into seven groups, six of which received intravenously either triple typhoid vaccine,[3] typhoid combined vaccine,[3] or the aliquots to reduce the standard errors of the counting rates below ±3 per cent.

The distribution of I^{131} did not differ notably among the seven groups of mice (Table 2). Enlargement of the spleen was present in all the mice, including the controls, but in no spleens was the administered I^{131} present in a concentration of more than 63 per cent of the concentration in the serum, and the average proportion was 39 per cent.

[3] The triple typhoid vaccine (Lederle) was stated to contain 100,000,000 bacteria per cubic centimeter in 0.45 per cent phenol. The typhoid combined vaccine (Lederle) was stated to contain 2,000,000,000 bacteria per cubic centimeter in 0.45 per cent phenol.

In a third group of experiments, again with ABC mice, certain parts of the first experiment were repeated. The mice, 230–90 days old, were bred here from A mice and C57 black mice.[4] Six males were injected intramuscularly, 16–29 days after tumor implantation, with labeled iodide (43 mμg. of added inert iodine in 0.01 cc. of solution per gram of body weight). They were killed 45 minutes later. Six control mice. The thyroid glands of the tumor mice contained proportionately less I^{131}, as compared with their serum I^{131} concentrations, than did those of the controls, in four of the six pairs of mice. The distribution of I^{131} among the other tissues was similar in tumor and control mice. The spleens of the tumor mice were enlarged as were, less markedly, their livers and kidneys.

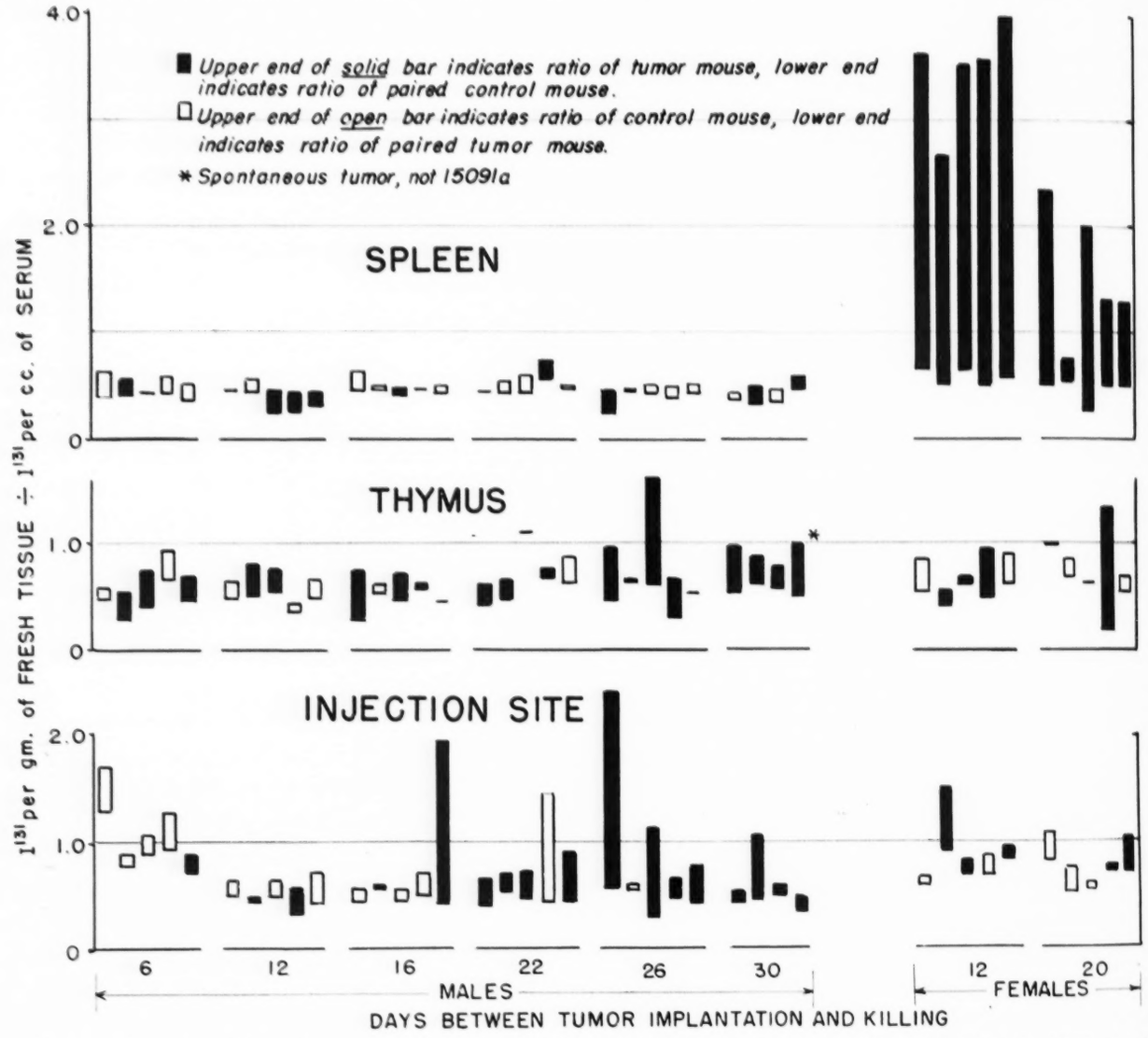

FIG. 2.—The ratios of tissue I^{131} concentration to serum I^{131} concentration for the spleens, thymus glands, and injection sites (hind leg) of the 58 male and 20 female ABC mice in the third experiment.

control mice (one with a spontaneous tumor) were similarly treated. This time, the thyroid glands and tracheas were also suspended in 1 per cent sodium hydroxide to give a 1–20 dilution and were treated like the other tissues.[5] Five of the six tumor mice had higher ratios of spleen I^{131} concentration to serum I^{131} concentration than did the paired

A larger series of animals was used next—58 male and 20 female ABC mice; the tumor was implanted subcutaneously in half of them, with the other half serving as controls. They were injected with iodide and killed 6–30 days after tumor implantation. Certain parts of the body, including

[4] Both strains were kindly supplied by Dr. Leonell C. Strong.

[5] Dr. Earl R. Miller of the University of California kindly suggested this change in technic to prevent excessive loss of beta rays through self-absorption in the dried tissue.

the entire hind leg into which iodide had been injected, were removed for measurement of I^{131}. Aliquots were measured until the standard errors of the counting rates were less than ±3 per cent.

The findings are presented in Figures 1, 2 and 3. The spleens of the male tumor mice and the control mice contained lower concentrations of I^{131} than did their sera. However, nine of the ten females with tumors had higher concentrations of I^{131} in their spleens than in their sera, although the concentrations of I^{131} in the spleens of the control females were normal. The thyroid glands of the tumor mice, both male and female, contained less I^{131} than did those of the controls, with few exceptions. The thymus glands of the tumor animals, in 24 of the 39 pairs of mice, contained higher proportions of I^{131} compared to the serum concentrations of I^{131}, than did the thymus glands of the controls. The thymus glands were generally smaller in the tumor mice than in the control mice. Concentrations of I^{131} in the serum and at the site of injection did not differ noticeably between the tumor and the control mice.

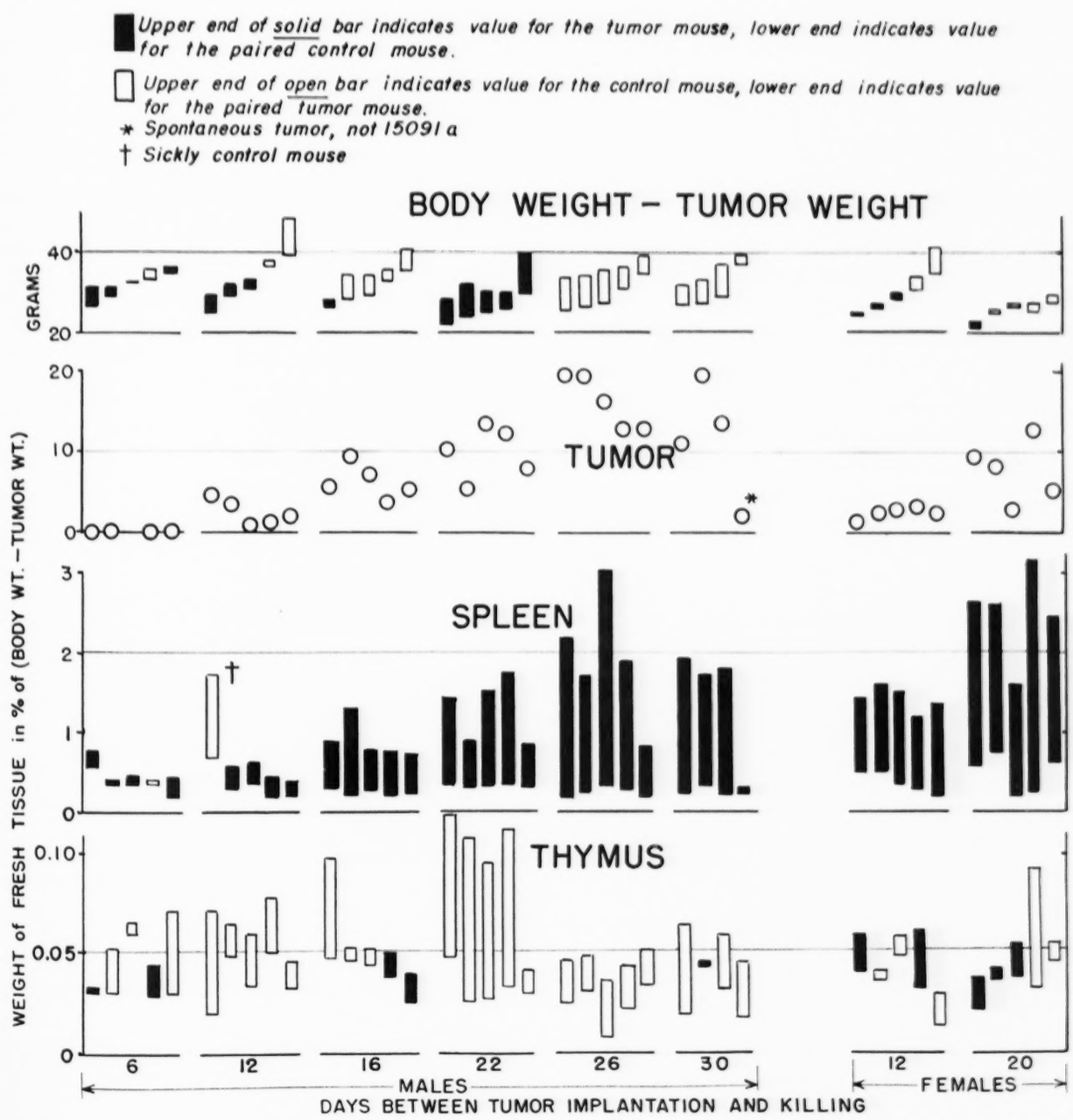

FIG. 3.—Body weights and fresh weights of the tumors, spleens, and thymus glands of the 58 male and 20 female ABC mice in the third experiment.

DISCUSSION

The differences in the distribution of the injected iodine among the tissues of tumor mice and the control mice were definite in the thyroid and the spleen. Unlike our findings in rats (8), the difference in the I^{131} content of the thyroids was not obviously related to any difference in the concentrations of I^{131} in the blood sera of the tumor mice and the control mice. In the two mice with spontaneous tumors, the quantities of I^{131} in their thyroids exceeded those found in the control mice. The tumors of these two mice were among the least necrotic of those seen in the entire group of 206 mice. How much of the difference in I^{131} content of the thyroids was due to differences in the size of the thyroids of the tumor mice and the control mice was not ascertained. The difference in the I^{131} concentrations of the spleens of tumor and control mice was inconstant in its occurrence. It was suggested (by Dr. Paul S. Nicholes of the University of Utah) that part of the I^{131} was occasionally attached, before injection, to some material which the spleens of tumor animals removed from the blood. Differences in the organ weights of tumor and control mice, similar to those found by others (6), were observed.

Several other features in the distribution of I^{131} among the tissues are of interest. According to studies of larger animals (3, 9, 10) this distribution may be largely confined to the extracellular fluid and so will presumably resemble that of chloride in most tissues. At present it is difficult to describe the recirculation of absorbed iodine through the extracellular fluid and other compartments of the various tissues from the limited data available. The absorption of the injected I^{131} was largely completed in 45 minutes, as demonstrated by the relatively low concentrations found in the injection site. Obvious exceptions to a chloride-like distribution of the absorbed I^{131} were in the thyroid and in the stomach. The concentrations of I^{131} in tissues other than the thyroid decreased concomitantly. Selective retention of I^{131} beyond the quantities presumed to be in the extracellular fluid was observed with certainty only in the thyroid and, occasionally, in the spleen. These findings were true of both the large and the small quantities of iodide. Similar but more rapid elimination of I^{131} from tissues other than the intestine was observed by Seligman *et al.* (5), who used larger quantities of iodide than were employed here. They gave approximately 21 μg. of iodine per gram of mouse; we gave quantities ranging from 0.0013 to 1.3 μg. per gram. For comparison, it may be estimated that 0.01 μg. of iodide is usually present per gram of serum or extracellular fluid. Whether the quantity of injected iodide was more or less than this made little difference in the relative proportions of I^{131} among the various extra-thyroid tissues.

The concentration of I^{131} in tissue, compared with that in serum, differed in mice and rats (8) in several instances: Mice had higher proportions of I^{131} in their thymus glands and lower proportions in their livers and ear pinnae. The tumors contained, on the average, 40–50 per cent as high a concentration of I^{131} as occurred in the blood serum, thus resembling, in this respect, Walker tumor 256 in rats (8). In general, the concentrations of I^{131} in the tumors exceeded those in the leg muscle, testes, and liver; equaled those in the heart and spleen (except as already discussed); and were lower than those in the serum, blood, kidneys, lungs, thymus, stomach, forestomach, and thyroid.

SUMMARY

Measurements were made of the distribution of I^{131} among the tissues of 206 mice killed either 45 minutes or 25 hours after injection of labeled sodium iodide in quantities varying from 0.0013 to 1.3 μg. of iodine per gram of mouse. Smaller quantities of I^{131} were found in the thyroid glands of mice with implants of tumor 15091a than in the thyroids of control mice, although the concentrations of I^{131} in their blood were similar. Higher concentrations of I^{131} were found in the spleens of tumor mice than in their blood, on some occasions. The explanation for this is uncertain. Splenic enlargement in mice without tumors was not a sufficient cause for such concentration of I^{131}. Compared to the concentrations of I^{131} in the sera, the livers and ear pinnae of mice contained lower concentrations of I^{131}, and their thymus glands contained higher concentrations than did the corresponding tissues in rats. Otherwise, the distribution of the injected iodide was similar in mice and rats.

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Influence of Age of Host and Ovaries on Tumorigenesis in Intrasplenic and Intrapancreatic Ovarian Grafts*†

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The formation of tumors in intrasplenic ovarian grafts in castrated hosts has been observed repeatedly in rats (1, 10) and mice (2, 7, 8, 9). In all the studies undertaken up to this time both young hosts and donors were used; in fact, autologous grafts have been made in most of the experiments.

The theory has been proposed that ovarian tumorigenesis occurs in the intrasplenic and intrapancreatic grafts in castrated animals because of the existing hormone imbalance due to a decreased systemic level of estrogen and an increased level of gonadotrophin (3, 7). Similar conditions might occur in old animals whose ovaries have lost most of their capacity to produce hormones. It is known, for example, that urinary gonadotrophins increase subsequent to the menopause. Under such conditions, why do not granulosa cell tumors occur more frequently, if the above theory is correct? Two possibilities present themselves: (*a*) The ovaries of old animals may be refractory to stimulation by even augmented amounts of gonadotrophin or (*b*) the level of gonadotrophin may be inadequate in quality or quantity. The former assumption seemed more likely, so experiments were designed to investigate this hypothesis.

MATERIALS AND METHODS

Male and female mice of the A, C57, and CBA strains were used.[1] They were maintained throughout life on Purina Fox Chow and water and in an air-conditioned room. Four groups of experiments were undertaken.

Group 1.—Ten mice were castrated when 8–14 days of age, and an ovary from a donor (17–34 days old) of the same strain was transplanted into the spleen when the hosts were 32–49 days old (Table 1).

Group 2.—Eighteen mice were castrated when 204–370 days old. Ovaries from mice 204–370 days old were implanted into the spleens immediately after removal of the gonads. Autologous grafts were made in the females (Table 2).

Group 3.—Ovaries from old donors (340–407 days) were transplanted into the spleens of six mice, from 54 to 105 days of age, and immediately subsequent to castration (Table 3).

Group 4.—Four mice of the C57 strain were castrated when 55 days old, and ovaries from donors 409–91 days old were implanted into the pancreas (Table 4).

All operations were undertaken without attempts to maintain asepsis but with care to prevent undue infection. Usually 5–15 minutes elapsed between removal of the ovary and its implantation. The ovaries were invariably traumatized during the procedure.

The mice were examined several times each week and were removed from the experiment only when large tumors were palpable or, with the exception of the animals in the first group, when their condition indicated impending death. At autopsy the following tissues were examined: mammary glands, ovarian graft, adrenal glands, pituitary gland, salivary glands, and the acessory genital organs. The presence or absence of adhesions of the grafts to the body wall or adjacent viscera was determined. The grafts, adrenal glands, and genital tissues were fixed in Bouin's fluid and after sectioning at 7 μ were stained in hematoxylin and triosin.

OBSERVATIONS

Tumors in intrasplenic ovarian grafts in mice castrated before puberty.—The mice were killed after they had carried the grafts from 180 to 187 days (Table 1). Only four of the ten mice had lesions considered, from histological examination, to be tumorous. Two of the nontumorous grafts were adherent to the body wall.

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[1] These mice were derived from animals furnished from the colony of Dr. L. C. Strong.

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The tumorous grafts were small and histologically not unlike those described previously (8, 9). A few follicles and hemorrhagic cysts were found in portions of the tumorous grafts. Luteinized atretic follicles, hemorrhagic follicles, and a few small and medium-sized ovarian follicles made up most of the nontumorous grafts. Ingrowth of the germinal epithelium was present in all the grafts were adherent to the body wall and because two of the hosts survived less than six months subsequent to intrasplenic implantation of the ovaries, the total number of satisfactory experiments was only ten. The average age of the four transplants that were tumorous was 299 days, and the average of the six nontumorous transplants, not excluded as mentioned above, was 280 days.

TABLE 1

TUMORS IN INTRASPLENIC OVARIAN GRAFTS IN MICE CASTRATED BEFORE PUBERTY

Number and strain	Sex	Age at castration (days)	Age at grafting (days)	Age of donor (days)	Age of graft at autopsy (days)	Age of host at autopsy (days)	Ovarian tumors	Uterine weight (mg.)
1 A	♂	13	49	26	180	230	get* 6×8×8.5 mm.	
2 A	♂	13	49	26	180	230	get 5×5×6 mm.	
3 C57	♂	8	34	34	185	219	None	
4 C57	♀	8	32	32	187	219	None	40
5 C57	♀	8	32	32	187	219	Adhesion	110
6 C57	♀	8	32	32	187	219	Mixed tumor 2×6×7 mm.	28
7 A	♀	12	47	26	180	227	None	34
8 A	♀	14	49	26	182	232	Mixed tumor (about 5 mm.)	34
9 A	♀	14	49	26	182	232	Adhesion	87
10 A	♀	11	39	17	182	221	Luteoma (about 5 mm.)	38

* get—granulosa cell tumor.

TABLE 2

TUMORS IN INTRASPLENIC OVARIAN GRAFTS IN MICE CASTRATED AT 204–370 DAYS OF AGE

Number and strain	Sex	Age at castration and grafting (days)	Age of donor (days)	Age of graft at autopsy (days)	Age of host at autopsy (days)	Wt. of uterus (mg.)	Ovarian tumors	Other tumors
9 CBA	♀	204	204	237	441	260.	None	
11 CBA	♀	204	204	428	632	238.8	None	Adenoma of adrenal Mammary tumor
17 CBA	♀	204	204	183	387	59.	Adhesion	Mammary tumor
21 CBA	♀	205	205	285	489	58.2	get*	
23 CBA	♀	204	204	423	625	132.5	Adhesion	
25 CBA	♀	209	209	371	580	46.	None	
26 CBA	♀	209	209	412	621	146.8	Adhesion	
10 CBA	♂	204	204	231	441		Adhesion	
14 CBA	♂	204	204	237	441		None	Hepatoma
16 CBA	♂	204	204	428	632		Adhesion	
22 CBA	♂	205	205	252	457		get	
13 A	♀	266	266	274	540		get	
18 A	♀	369	369	226	595	28.	None	Lung adenoma
24 A	♀	310	310	97	407	30.	None	
19 A	♂	286	369	355	565		get	
30 A	♂	268	346	192	460		None	
31 A	♂	370	340	160	520		None	

* Granulosa cell tumor.

except the two that were adherent to the body wall.

The seminal vesicles of all the male hosts were atrophic. The uteri of all mice without parietal adhesions were atrophic.

Ovarian grafts in adult mice.—Only four of the seventeen mice, which were 204–307 days old when they were castrated and when the intrasplenic ovarian grafts were made, had granulosa cell tumors (Table 2). Because five of the transplants

The granulosa cell tumors were not unlike those described previously (8, 9). They were composed predominantly of massive and trabecular arrangements of granulosa cells. Normal follicles were lacking in these grafts. Cells laden with lipoid and vacuolated cells were found in portions of most tumors. The nontumorous grafts consisted fundamentally of an ingrowth of cells from the germinal epithelium and of stroma.

The uteri of several of the mice were quite large.

The uteri of untreated ovariectomized mice rarely weighed more than 40 mg. Those animals with adhesions usually had hypertrophic uteri (see 23 CBA and 26 CBA in Table 2). One mouse with a large uterus (11 CBA) had a small adenoma of the adrenal cortex. Such tumors produce substances that stimulate the female genital tissues (4, 11). Another mouse (9 CBA) had no adrenal lesions of the hypertrophic type, and, apparently, adhesions to the parietes were lacking. The graft was adherent to the pancreas and mesenteries. Venous drainage may have been established through the mesometrium, although adhesions to this area were not noted at necropsy.

areas of hemangiomatous tissue were present in all of them. Spicules of bone were found in one. The tumorous areas were composed of masses of trabecular or follicular arrangements of granulosa cells. Ingrowths of the germinal epithelium were slight, but such observations are not uncommon in the larger tumors.

All the four castrated mice of the C57 strain that had ovaries from donors 409–91 days old, transplanted intrapancreatically when they were 55 days old, had luteomas or granulosa cell tumors when they were 320–69 days old (Table 4). The tumors were not unlike those described previously (8, 9).

TABLE 3

TUMORS IN INTRASPLENIC OVARIAN GRAFTS IN YOUNG CASTRATED MICE BEARING OVARIES FROM OLD MICE

Number and strain	Sex	Age at castration and grafting (days)	Age of donor (days)	Age of graft (days)	Age of host at autopsy (days)	Ovarian tumor
27 A	♂	54	407	303	367	get* 16×10.5 mm.
28 A	♂	54	407	337	391	get 18×16 mm.
29 A	♂	54	346	387	441	get 8×5×4 mm.
32 A	♂	105	340	388	493	get 10×8×7 mm.
34 A	♂	105	370	388	493	Adhesion
33 C57	♂	55	375	152	207	None

* Granulosa cell tumor.

TABLE 4

OVARIES FROM OLD MICE OF THE C57 STRAIN TRANSPLANTED INTO THE PANCREAS OF YOUNG CASTRATED MICE OF THE SAME STRAIN

No.	Sex	Age of host at operation (days)	Age of donor (days)	Age of host at death (days)	Age of graft at death (days)	Ovarian tumor
34	♀	55	491	369	313	Luteoma
35	♂	55	409	320	265	get* 21.1×14 mm.
36	♂	55	409	320	265	get 21×14 mm.
37	♀	55	409	369	313	Mixed† 10×10.4 mm.

* Granulosa cell tumor.

† Mixed—granulosa cell tumor and luteoma.

Two of the female mice of the CBA strain had mammary adenocarcinomas. Such tumors are not uncommon among untreated multiparous females or estrogen-treated mice of this strain and in this laboratory.

Old ovaries transplanted into young hosts.—Six mice were castrated when 54–105 days of age, and ovaries from mice 340–407 days old were transplanted into their spleens. Four of the hosts survived more than 180 days and had no adhesions to the parietal peritoneum (Table 3). The average age of the four mice bearing the granulosa cell tumors were 423 days, and the ovaries were, on the average, 729 days old.

The tumors were somewhat different than those arising in younger ovaries. Areas of necrosis or

DISCUSSION

Old ovaries, transplanted intrasplenically or intrapancreatically into young castrated mice, gave origin to tumors with about the same incidence as did young ovaries, transplanted into young hosts. The number of observations is too limited to determine whether or not the incidence of tumors would be lower when old ovaries are transplanted but indicate that the difference is not great. The old ovary is apparently as susceptible, or as nearly so, as is the younger ovary, when both are in the young environment.

A satisfactory experiment cannot be attained when old ovaries are transplanted into aged (400–500 days old) animals, because of the restricted survival of the older hosts. However, when castrated

mice from 200 to 300 days of age received transplants from donors of a similar age, the incidence of granulosa cell tumors was low—four of ten mice had tumors at death, whereas eight of ten young mice bearing old ovaries had tumors in the transplants.

The hormonal environment of old and largely anovular ovaries in aged mice is not compatible with the origin of many spontaneous, granulosa cell tumors, as these tumors rarely occur (5). Occasionally an old mouse will have ovaries showing ingrowths of the germinal epithelium similar to those seen in the pretumorous stages in the intrasplenic ovarian transplants in castrated hosts. Whether or not these might become tumorous if the animals had survived for a longer period is problematic. To account for the failure of tumors in the old, intact ovaries, it must be assumed that, should the hypothesis be correct as stated in the introduction, the production of the gonadotrophic hormone must be quantitatively or qualitatively inadequate. This assumption is compatible with the observations in the present experiments. Both the observations on the experiments described above and the assumed reason for the rarity of spontaneous ovarian tumors indicate that age changes in the pituitary coincide with age changes in the ovaries.

The low incidence of ovarian tumors in the mice that were castrated when very young and in those that received intrasplenic transplants several weeks later (five of eight mice without adhesions) differs from that observed in many mice that were castrated and that received transplants of ovaries on the same day; but all the mice were killed when the grafts were no older than 232 days. In an earlier study (9), 21 of 22 mice with intrasplenic ovarian transplants had tumors when the grafts were 278 days old.

SUMMARY AND CONCLUSIONS

Five of eight mice that were castrated when 8–14 days of age and that received intrasplenic transplants of ovaries when 32–49 days of age had ovarian tumors when the grafts were 219–30 days old.

Four ovarian tumors appeared in intrasplenic or intrapancreatic transplants of ovaries from ten mice that were 204–70 days old when the ovaries were transplanted intrasplenically into castrated hosts of a similar age; in these the grafts were not adherent to the parietes.

Eight ovarian tumors appeared in intrasplenic or intrapancreatic grafts of ovaries from mice 340–491 days of age when they were transplanted into mice from 54 to 105 days of age.

Old ovaries are susceptible to tumorigenesis when in the proper environment and apparently as responsive as are the ovaries of younger animals.

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Anti-leukemic Assays on Certain Pyrimidines, Purines, Benzimidazoles, and Related Compounds*

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Much of the fundamental biochemical effort in the cancer field has been directed toward gaining a better understanding of the role of nucleic acids in the normal and neoplastic cell. The rationale behind such studies may be partially summarized in the words of Spiegelman and Kamen (1): ". . . it is supposed that genes control ultimately all cellular functions, including protein synthesis. From the close association of genes with nucleoproteins, it is natural to assume that nucleic acids may be involved in protein synthesis. Such an assumption can be supported by the fact that nucleic acids are always found associated with self-duplicating units (e.g., genes, viruses, plastids, transforming principals of pneumococci and coli)." Since, from a genetic standpoint, cancer is a mutant strain of cells with one or more genes or systems excessively promoting (or failing to inhibit) growth, a logical point for chemotherapeutic attack might be the metabolic system utilizing the constituents of which the normal and cancer chromosomes are largely composed, e.g., nucleic acids and histones. If there is reason to believe that the nucleic acid metabolism (and in turn purine or pyrimidine metabolism) of the normal and the mutant cancer cell differ in certain respects, then a search for compounds which might inhibit that part of the system peculiar to the cancer cell is a worth-while endeavor.

The recent reports of the anti-leukemic activity of 2,6-diaminopurine, an antagonist of pteroylglutamic acid and adenine in the metabolism of *Lactobacillus casei* (2), and the tumor-inhibiting activity of 5-amino-7-hydroxy-1*H*-*v*-triazolo [*d*] pyrimidine, a powerful purine inhibitor (3), have emphasized the importance of searching for other inhibitors in the nucleic acid metabolic system. Such findings as are mentioned above not only offer compounds worthy of testing against various types of human neoplasia but also aid in our understanding of the differences in normal and neoplastic cells.

Woolley (4) has reported that benzimidazole is antagonistic to amino-purines in certain microorganisms and that its growth inhibition can be overcome by adenine or guanine.

The present study involves the screening of a number of pyrimidines, purines, benzimidazoles, and related compounds against a strain of mouse leukemia known to respond to aminopterin (4-aminopteroylglutamic acid) and to 2,6-diaminopurine.

EXPERIMENTAL

The synthesis of the compounds involved in the present study will be described elsewhere. Compounds Nos. 88, 122, 124, and 82 were commercial samples. All others were prepared in this laboratory.

The procedure used for screening compounds for toxicity and anti-leukemic action has been described in an earlier report (5). In all experiments, candidate compounds have been injected intraperitoneally at the maximum tolerated dose, every other day, for a total of 10 injections, starting on the second day after leukemic inoculation. Results are reported in terms of average survival times of groups of ten mice as compared to concurrently run untreated controls and aminopterin-treated controls. These data are summarized in Table 1 and are further compared in Figure 1.

DISCUSSION

It is interesting to note that 2,4,5,6-tetraminopyrimidine, which contains the 2,4-diaminopyrimido portion common to most anti-folic acid compounds, and which is the pyrimidine used in *in vitro* synthesis of 4-amino-pteroylglutamic acid, showed no anti-leukemic activity.

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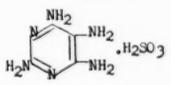
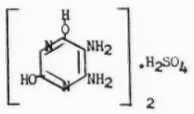
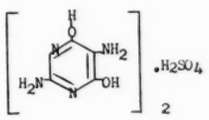
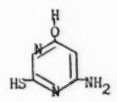
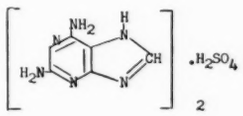
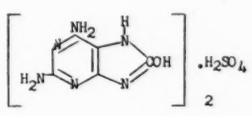
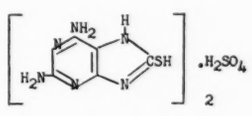
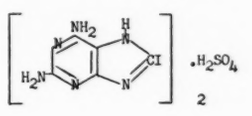
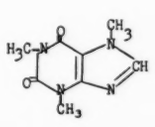
We have confirmed the finding of the anti-leukemic activity of 2,6-diaminopurine in strain Ak 4 mouse leukemia reported by Burchenal, *et al.* (2). However, in our limited experiments, this purine has not been as active as 4-aminopteroylglutamic acid. This difference is probably due to our failure to use the most effective dose of 2,6-diaminopurine. The Sloan-Kettering group (2) stated that 4-amino-N^{10}-methylpteroylglutamic acid was active over a slightly greater dosage range than was 2,6-diaminopurine. It appears that substitution at the 8-position of 2,6-diaminopurine lowers or negates the activity of this compound with regard to anti-leukemic action. The 2,6-diamino-8-hydroxy- and the 2,6-diamino-8-mercaptopurine, as well as the 2,6-diamino-8-iodopurine, showed no significant activity.

The benzimidazoles, 5-nitrobenzotriazole, in-

TABLE 1

THE RESULTS OF ANTI-LEUKEMIC ASSAYS ON CERTAIN PYRIMIDINES, PURINES, BENZIMIDAZOLES, AND RELATED COMPOUNDS

S.R.I. No.	Compound	Structure	Maximum tolerated dose (mg/kg)*	Average survival time (days)	Per cent increase in survival time	90-day survival	R.C.I.†
108	2,4,5,6-Tetraminopyrimidine sulfite		97	11.0	−12.7	0/10	−0.24
116	2,6-Dihydroxy-4,5-diaminopyrimidine sulfate		63	12.1	− 4.7	0/10	−0.09
117	2,5-Diamino-4,6-dihydroxypyrimidine sulfate		7.8	11.9	− 6.3	0/10	−0.13
118	2-Mercapto-4-amino-6 hydroxypyrimidine		125	11.6	− 8.7	1/10	−0.17
135	2,6-Diaminopurine sulfate		100 125	12.6 12.9	+29.9 +29.0	0/10 0/10	+0.73 +0.48
149	2,6-Diamino-8-hydroxypurine sulfate‡		125	9.9	+ 2.1	0/10	+0.05
150	2,6-Diamino-8-mercaptopurine sulfate		125 62.5	8.9 9.4	− 8.2 − 6.0	0/9 0/5	−0.20 −0.10
152	2,6-Diamino-8-iodopurine sulfate		50 25	9.5 9.5	− 2.1 − 5.0	0/10 0/10	−0.05 −0.08
88	Caffeine		62.5	10.5	−16.7	0/10	−0.31

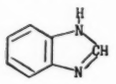
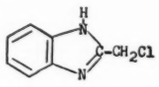
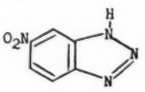
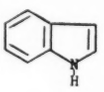
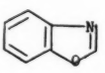
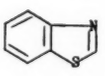
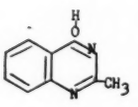
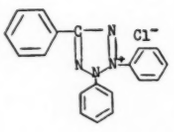
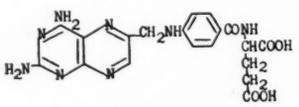
* Maximum tolerated dose, an approximate value.

† Relative chemotherapeutic index = $\frac{\text{Per cent increase in life span of group treated with a given compound}}{\text{Per cent increase in life span of aminopterin-treated group}}$.

‡ Impure sample.

NOTE: Animals surviving 90 days not included in calculations of survival time.

TABLE 1—*Continued*

S.R.I. No.	Compound	Structure	Maximum tolerated dose (mg/kg)*	Average survival time (days)	Per cent increase in survival time	90-day survival	R C.I.†
122	Benzimidazole		250	11.8	+ 7.3	0/10	+0.12
98	2-Chloromethylbenzimidazole		44	11.2	−11.8	1/10	−0.23
154	5-Nitrobenzotriazole		62.5	9.7	− 3.0	0/10	−0.05
124	Indole		125	10.3	− 4.6	0/10	−0.09
129	Benzoxazole		3.9	9.3	− 4.1	0/10	−0.10
130	Benzothiazole		31.3	10.9	+ 0.9	1/10	+0.02
113	2-Methyl-4-hydroxyquinazoline		250	10.4	−18.1	1/10	−0.36
82	2,3,5-Triphenyltetrazolium chloride		7.8	11.3	−13.7	1/9	−0.40
114	4-Aminopteroylglutamic acid (aminopterin)		0.23	20.4 19.1 17.5 16.1 13.7 19.1 16.0	+65.8 +50.4 +59.1 +49.1 +41.2 +59.2 +60.0	1/10 0/10 0/10 2/10 0/9 0/10 0/10	

* Maximum tolerated dose, an approximate value.

† Relative chemotherapeutic index = $\frac{\text{Per cent increase in life span of group treated with a given compound}}{\text{Per cent increase in life span of aminopterin-treated group}}$.

NOTE: Animals surviving 90 days not included in calculations of survival time.

dole, benzoxazole, benzothiazole, 2-methyl-4-hydroxyquinazoline, and 2,3,5-triphenyltetrazolium chloride were all negative when assayed against transplanted Ak 4 leukemia in mice.

SUMMARY

A number of compounds of interest as possible purine or pyrimidine antagonists have been assayed for anti-leukemic action against transplanted lymphoid leukemia (Ak 4) in mice.

The pyrimidines studied were uniformly inactive.

Of the 2,6-diaminopurine derivatives assayed, only the parent compound definitely increased survival time. Introduction of a hydroxy, mercapto, or iodo group at the 8-position of 2,6-diaminopurine apparently inactivates this anti-leukemic molecule.

Benzimidazole, 2-chloromethylbenzimidazole, 5-nitrobenzotriazole, indole, benzoxazole, benzothiazole, 2-methyl-4-hydroxyquinazoline, and 2,3,5-triphenyltetrazolium chloride were screened but showed no anti-leukemic activity.

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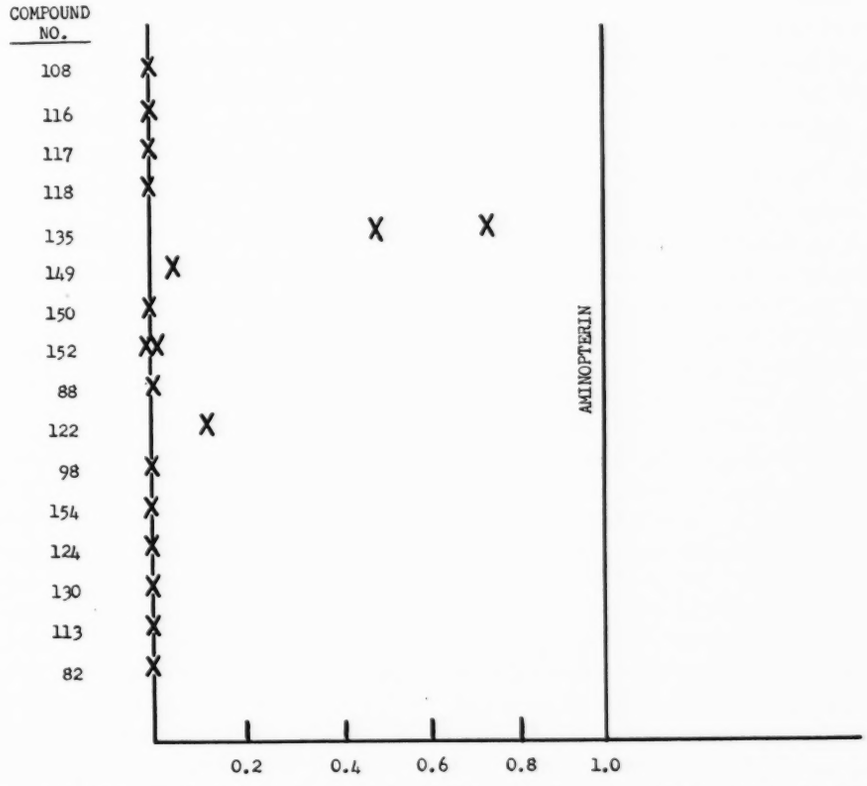

FIG. 1.—Relative chemotherapeutic index of certain pyrimidines, purines, benzimidazoles, and related compounds with aminopterin (4-aminopteroylglutamic acid) as the standard of reference.

The Effect of 5-Amino-7-Hydroxy-1*H*-*v*-Triazolo (*d*) Pyrimidine (Guanazolo) on a Variety of Neoplasms in Experimental Animals*

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In a systematic search for chemical agents which might act synergistically with the sulfonamides in inhibiting bacterial growth, Roblin and his associates synthesized analogs of guanine, adenine, xanthine, and hypoxanthine, in which a nitrogen replaced a carbon atom of the corresponding purine (4). Of the triazolopyrimidines studied by this group, 5-amino-7-hydroxy-1*H*-*v*-triazolo (*d*) pyrimidine was found to be the most potent purine inhibitor, using the growth of *E. coli* and *S. aureus* as indicators. Kidder, using this same guanine analog, demonstrated marked inhibition of the growth of the animal microorganism *Tetrahymena geleii* (2). This latter observation, which was a part of an exhaustive study made by Kidder and his associates on the growth requirements and metabolic pathways in *Tetrahymena*, constituted a preliminary to the exploration of the effect of 5-amino-7-hydroxy-1*H*-*v*-triazolo (*d*) pyrimidine (guanazolo, triazolopyrimidine) on cancer cells. Recently, Kidder's group reported that guanazolo had an inhibitory effect on two mouse breast cancers and a lymphoid leukemia in mice (3). Since the first three tumors studied had all been controlled effectively without significant toxicity to normal cells, Kidder concluded tentatively that a fundamental biochemical difference in purine metabolism existed between normal and neoplastic cells. He further suggested that the pathway of purine metabolism peculiar to the latter might be similar for all cancerous tissue, thus supporting Greenstein's speculation that all malignant neoplasms resemble each other more than normal cells do, and, therefore, an agent which could control one type of cancer would probably control all others.

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It is the purpose of this report to present further evidence on the effect of the triazolopyrimidine on five transplantable mouse tumors, on the Brown-Pearce carcinoma in rabbits, and on leukemia 9417 in Ak mice. It will be shown that the compound has striking carcinostatic activity against some of the tumors but no demonstrable effect on others.

MATERIAL AND METHODS

The triazolopyrimidine was synthesized, with minor modifications, according to the method of Roblin *et al.* (4). The purity was checked spectroscopically, and a sample obtained from Dr. Kidder, analyzed by this method, indicated that the two materials were identical.

The mouse tumors were Sarcoma 180 in Paris RIII and C57 mice; 755 tumor in C57 mice; RC tumor in dba mice; Eo771 in C57 mice; leukemia No. 9417 in Ak mice; 6C3HED in C3H mice. The types of tumors designated by the above symbols are shown in Table 7. The solid tumors were transplanted by the trocar method, placing the fragment in the axillary region on one side. The leukemia was transmitted by intraperitoneal injection of a saline suspension of splenic tissue from a leukemic donor. The growth of the tumors was checked at frequent intervals by caliper measurements and recorded, but the objective evidence for the presence or absence of an effect of the triazolopyrimidine was based on the wet weights of the tumors determined to the nearest milligram. In the experiments with the transmissible leukemia and the transplantable lymphosarcoma (6C3HED), the objective end-point used for the evaluation of the therapeutic effect of the triazolopyrimidine was the survival of the treated animals, compared with that of the untreated controls. The Brown-Pearce carcinoma was transplanted bilaterally to the anterior chamber of the eye of rabbits. This proved to be a very convenient method of determining the effect of the chemical agent on this tu-

mor, inasmuch as the viability of the transplant could be easily recognized by direct observation, and the growth of the transplants was very uniform, completely filling the anterior chamber of untreated animals in 7–10 days. In these experiments, the growth of the transplant was recorded daily by direct caliper measurements, and, at the termination of the experiment, the rabbit was sacrificed and the tumor, together with the attached iris, was removed and weighed to the nearest milligram. The duration of the experiments with the Brown-Pearce carcinoma was determined by the rate of growth of the tumor in the untreated control animals; the observations were terminated when the tumor filled the anterior chambers of the control animals, because delay beyond this point was followed by rupture of the tumor through the cornea and hemorrhage which made accurate weighing of the tumor impossible.

The mice and rabbits were given a stock diet of Rockland mouse and rat pellets, respectively. Water was allowed *ad libitum*.

Because of the relative insolubility of the triazolopyrimidine in water, suspensions of it in isotonic saline or in 10 per cent gum acacia were tried in preliminary experiments. These resulted in a variation in both therapeutic and toxic effects. It was found that a soluble, stable sodium salt of the triazolopyrimidine could be prepared conveniently by the addition of 1 part of 0.1 N NaOH to 2 parts of an aqueous suspension, which was heated gently in a water bath. By this means, a stock solution containing 400 mg. per 100 milliliters was prepared with a final pH which ranged between 9 and 10. A stock solution of dilute NaOH of pH 9.5 was prepared and was injected into the control animals in volumes comparable to those of the administered triazolopyrimidine solution. In all of the experiments, the route of administration was intraperitoneal.

TABLE 1

EFFECT OF THE TRIAZOLOPYRIMIDINE ON THE 755 TUMOR IN C57 MICE

Exp. no.	Group	No. of animals	Initiation of therapy after transplantation (days)	Dose of triazolopyrimidine (mg/kg)	Duration of therapy (days)	Mean tumor weight (mg.)	Standard deviation (mg.)	Standard deviation of mean (mg.)	Av. body weight (gm.) Beginning	End
5	Control	23	1			735	397	83	18.2	20.2
	Treated	21		50 once daily	20	83	73	16	19.5	18.4
19	Control	9				360	240	85	17.3	18.4
	Treated	15	1	50 once daily	17	34	30	8	18.5	19.3
24	Control	10				354	202	67	19.4	20.9
	Treated	10	1	50 once daily	13	36	17	6	17.4	17.6
	Treated	10	1	50 twice daily	13	4	1.5	0.5	17.7	14.4
16	Control	14				2,500	965	258	16.7	20.4
	Treated	22	22	12.5 thrice daily	9	1,600	725	154	19.1	19.5
22	Control	10				645	270	85	20.8	23.3
	Treated	10	8	50 twice daily	8	68	23	8	20.4	20.9
35	Control	10	9*			42	15	5		
	Control (saline)	10				280	151	50	18.8	19.5
	Control (NaOH)	15				307	125	34	17.8	18.9
	Treated	10	9	50 twice daily	6	45	51	17	18.6	19.0
36	Control	10	10*			52	35	12		
	Control (saline)	10				567	387	129	17.9	19.6
	Control (NaOH)	10				848	411	137	18.0	19.8
	Treated	10	10	50 twice daily	8	111	114	38	18.7	18.9
36 A	Control	5				6,384	1,470	735	19.2	25.0
	Treated	5	10	50 twice daily×13 50 once daily×14	27	391	54	27	19.2	19.0

*Sacrificed to determine mean tumor weight at initiation of therapy.

RESULTS

755 tumor in C57 mice.—The results of experiments utilizing this tumor are summarized in Table 1. From the information included in this and all subsequent tables, it is possible to estimate with accuracy the actual protocols for the individual experiments, even though the pertinent results are here summarized as averages; the standard deviation has been calculated in each instance in order to describe the range and the distribution of the individual tumor weights based on the mean tumor weight. The standard deviation of the mean which is necessary for the determination of statistical significance between two means is derived

from the standard deviation divided by the square root of the number of observations.

There are several points to be emphasized: (*a*) As can be seen from the results, the triazolopyrimidine has inhibited the growth of this tumor in every experiment, and mathematical analysis demonstrates that the difference between the mean tumor weights of the control and treated animals are statistically significantly different in each instance.

(*b*) The inhibitory effect of the compound on this tumor is demonstrable, whether administration is initiated shortly after transplantation, as in the first three experiments summarized, or after the tumor has become well established as shown in the last five experiments.

(*c*) In experiments 35 and 36 one set of control animals was sacrificed to obtain an estimate of the tumor weights in all animals at the outset of therapy. As can be seen from the results of this experiment, there was little further growth of the tumors after the triazolopyrimidine was administered, as compared to that in the untreated animals. It is to be emphasized, however, that there was no regression of the tumor.

(*d*) Because of the pronounced effect of the triazolopyrimidine on the 755 tumor, it was considered essential to rule out any effect of the alkaline solution on tumor growth. For this reason, as is summarized in experiments 35 and 36, both saline and dilute NaOH were administered to groups of animals, and, as can be seen, there was no significant difference among the tumor weights at the termination of the experiment.

(*e*) In experiment 24, the effect of the compound on the 755 tumor was observed when it was given once and twice each day. It is of interest to note that twice-daily administration resulted in a significantly greater inhibition of tumor growth than was observed following once-daily doses.

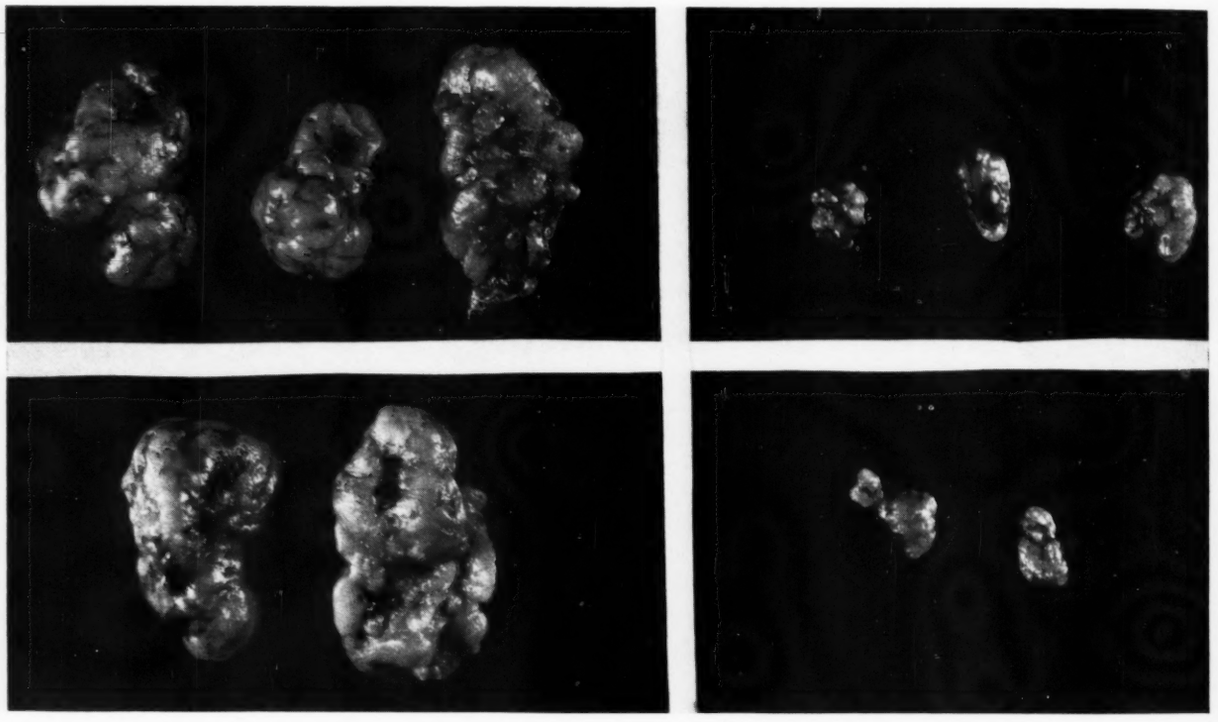

FIG. 1.—Effect of triazolopyrimidine on established 755 tumor in C57 mice. See Table 1, Exp. 36 A for complete protocol. Photographs ($\frac{2}{3}$ actual size) taken of control and treated tumors 27 days after onset of therapy. Treatment was not started until 10 days after transplantation of the tumor.

(*f*) In experiment 36 A, the triazolopyrimidine therapy was continued over a protracted time period, to determine whether or not the neoplasm would become refractory to the effects of the chemical agent. During the period of observation, there was no indication of "escape" of the tumor. Sequential graphings of the tumor in the treated animals showed progressive but very slow growth, with no apparent alteration of the curve during the experiment. The striking difference in the weights of the tumors in control and in treated animals, shown in Table 1, is equally clearly seen in photographs of the tumors reproduced in Figure 1.

(*g*) The final two columns of Table 1, which indicate the average body weight of the mice at the

beginning and end of the experiment, provide an over-all indication of the toxicity of the triazolopyrimidine at the doses employed. Although in two of the experiments there was some weight loss of the treated animals, in the remaining eight the treated animals maintained or gained body weight.

There is one additional important experiment which was carried out with this tumor which does not lend itself to tabular summarization. In a series of ten animals, the triazolopyrimidine injections were started 1 day following transplantation of the tumor and continued for 14 days. At this time, the very small tumors were removed under aseptic conditions and transplanted into untreated C57 recipients. Although the tumor growth in the treated mice had been inhibited exactly as in the experiments detailed in the table, in every instance following transplantation these tumors "took" and grew in the usual way.

In all the experiments, the tumors from both control and treated animals were examined histologically. Areas of necrosis within some of the tumors of the treated series were not considered significant, inasmuch as spontaneous necrosis occurred with equal or even greater frequency in the untreated controls. No consistent cytological differences could be distinguished in the tumors from the control and experimental series.

RC tumor in dba mice.—The results of the triazolopyrimidine therapy on this tumor are summarized in Table 2. As it can be seen, the chemical agent significantly inhibited this tumor without evident toxicity to the host, as indicated by the absence of weight loss in the treated animals. The inhibitory effect was apparent when therapy was instituted, shortly after transplantation (Exps. 4, 28), as well as after the tumor had become well established (Exp. 48). It is also to be noted that twice-daily administration of the compound accentuated the tumor inhibition. Histological study of the tumors from treated and from control mice failed to reveal distinguishing morphological differences.

Sarcoma 180 in Paris RIII and C57 mice.—Multiple observations on Sarcoma 180 were made, because this tumor is so frequently employed in screening experiments for potentially useful carcinolytic chemotherapeutic agents. The results are summarized in Table 3. In no instance was significant inhibition of the tumor observed. A single

TABLE 2

EFFECT OF THE TRIAZOLOPYRIMIDINE ON THE RC TUMOR IN DBA MICE

Exp. no.	Group	No. of animals	Initiation of therapy after transplantation (days)	Dose of triazolopyrimidine (mg/kg.)	Duration of therapy (days)	Mean tumor wt. (mg.)	Standard deviation (mg.)	Standard deviation of mean (mg.)	Average body weight (gm.) Beginning	End
4	Control	23				1,090	380	79	16.9	19.7
	Treated	18	1	50 once daily	16	313	131	32	17.3	18.1
28	Control	10				778	132	44	18.0	20.3
	Treated	10	1	50 once daily	11	340	95	32	17.8	18.4
	Treated	10	1	50 twice daily	11	137	53	18	16.4	16.5
48	Control	9				1,293	430	152	13.8	15.2
	Treated	10	7	50 twice daily	7	709	162	54	14.6	14.9

TABLE 3

EFFECT OF THE TRIAZOLOPYRIMIDINE ON SARCOMA 180 IN PARIS RIII AND C57 MICE

Exp. no.	Group	No. of animals	Initiation of therapy after transplantation (days)	Dose of triazolopyrimidine (mg/kg)	Duration of therapy (days)	Mean tumor wt. (mg.)	Standard deviation (mg.)	Standard deviation of mean (mg.)	Average body weight (gm.) Beginning	End
6	Control	13				1,470	610	169	16.4	19.0
	Treated	8	1	50 once daily*	14	980	358	136	21.8	19.4
9	Control	20				236	140	32	18.6	20.0
	Treated	17	1	12.5 once daily	10	261	82	20	19.9	17.0
9 A	Control	10				370	162	51	21.2	21.9
	Treated	10	1	25 once daily	8	362	194	61	22.0	25.0
13	Control†	10				335	132	44	19.0	19.8
	Treated	10	1	12.5 once daily	9	295	85	28	19.4	19.5
37	Control	10				179	88	29	23.7	23.5
	Treated	10	1	50 once daily	9	309	119	40	24.1	23.0
	Treated	10	1	50 twice daily	9	394	138	46	24.0	22.0

* A saline suspension was used in this experiment.

† C57 mice were used in this experiment.

experiment was conducted utilizing C57 mice to make certain that the negative result could not be due to differences in the host; however, here, too, no tumor inhibition was noted. No significant morphological effects on the tumors from treated animals could be detected.

tumor fragments were vascularized by vessels growing from the iris, and the viability of the fragments was unmistakable. In the untreated animals, the growth of the fragments was uniform and rapid, with complete filling of the anterior chamber occurring in about 10 days after the trans-

TABLE 4

EFFECT OF THE TRIAZOLOPYRIMIDINE ON ANTERIOR CHAMBER TRANSPLANTS OF BROWN-PEARCE CARCINOMA IN RABBITS

Exp. no.	Group	No. of anterior chamber tumor transplants	Initiation of therapy after transplantation (days)	Dose of triazolopyrimidine (mg/kg)	Duration of therapy (days)	Mean tumor wt. (mg.)	Standard deviation (mg.)	Standard deviation of mean (mg.)	Average body weight (kg.) Beginning	End
27	Control	9				786	258	91	2.71	2.60
	Treated	8	3	75 once daily	6	185	49	19	2.80	2.31
33	Control	8	3*			81	12	5		
	Control	10				515	75	25	2.59	2.56
	Treated	10	3	75 once daily	6	96	9.7	3.2	2.55	2.17
41	Control	8				522	105	40	2.74	2.72
	Treated	6	3	12.5 once daily	7	419	77	34	3.05	2.94
	Treated	6	3	25 once daily	7	209	41	18	2.96	2.79
	Treated	6	3	50 once daily	7	156	22	10	2.77	2.27

* Animals sacrificed to determine mean tumor weight at initiation of therapy.

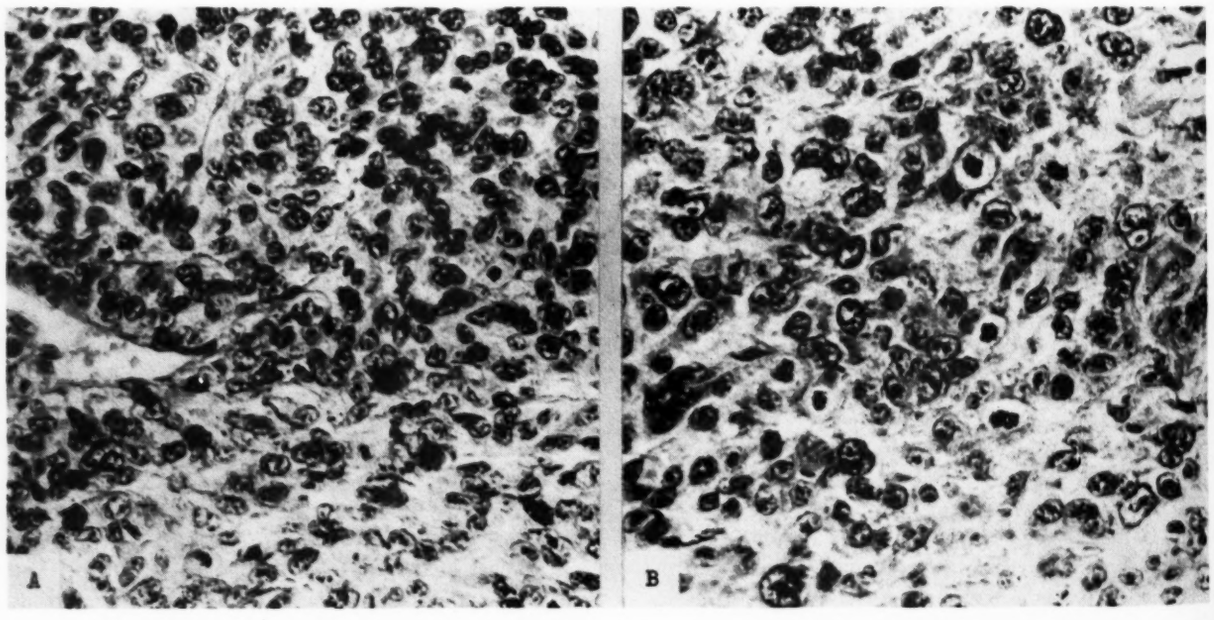

FIG. 2.—The effect of the triazolopyrimidine on the morphology of the Brown-Pearce carcinoma. *A* is a photomicrograph of the tumor from an untreated animal. *B* shows the histological characteristics of the tumor in a rabbit treated for 7 days with the triazolopyrimidine. See the text for a discussion of the differences. Magnification of both sections 400×.

Brown-Pearce carcinoma in rabbits.—The results of the triazolopyrimidine therapy on transplants of the Brown-Pearce tumor in the anterior chamber of rabbits' eyes are summarized in Table 4. In all instances therapy was not started until 3 days after transplantation. At this time, the plantation. The tabulated results indicate that significant tumor inhibition occurred at all dose levels tried, except 12.5 mg/kg/day. In experiment 33, a number of animals were sacrificed on the day that administration of the triazolopyrimidine was started, to have a quantitative esti-

mate of the tumor weights at the outset of therapy. As can be seen there was little further increase of the tumor weight after the onset of treatment with the triazolopyrimidine.

Morphological study of the tumors from treated and control animals uniformly revealed differences which distinguished the sections. Figure 2 illustrates the typical histological appearance of tumors from the two groups of animals. There are several features which are noteworthy: (*a*) The number of cells in the section from a treated animal are less than in the control—this is not the result of selection of a particular microscopic field but was apparent in all of the sections studied; (*b*) the size of the cells in the treated tumor is distinctly larger than those of the control tumor, and occasional giant cells are seen; (*c*) there is condensation of the nuclear material in irregular clumps. It is impossible, at this time, to interpret the significance of the morphological changes associated with the triazolopyrimidine therapy; they are being reported, however, as additional objective evidence of the effect of the compound on this tumor.

Eo771 tumor in C57 mice.—Table 5 summarizes the observations made on this tumor. It is evident, from the results, that the triazolopyrimidine exhibits carcinostatic activity against the tumor when administered either shortly after transplantation or after the neoplasm has become well established. There are two interesting points to be noted in Experiment 45. Nine days after the transplantation of this carcinoma, the mean tumor weight was approximately 250 mg. In the next 5 days, the tumor quadrupled in size in the untreated controls. This provides an indication of the rapidity of growth of this tumor. It is further of interest that, in spite of the high growth potentialities of the neoplasm, the trizaolopyrimidine showed significant inhibitory effects on the tumor, even though treatment was given for only 5 days.

As was the case with the mouse tumors previously described, no significant morphological differences between the control and the treated neoplasms could be detected.

Leukemia 9417 in Ak mice and 6C3HED tumor in C3H mice.—The results of the chemotherapy of these two tumors with the triazolopyrimidine are summarized in Table 6. The end-point for assessing the activity of a chemical agent against these experimental neoplasms is the comparison of the survival periods after transplantation between control and treated mice. As can be seen from the table, the triazolopyrimidine failed to prolong significantly the survival of treated mice with either the leu-

TABLE 5

EFFECT OF THE TRIAZOLOPYRIMIDINE ON THE Eo771 TUMOR IN C57 MICE

Exp. no.	Group	No. of animals	Initiation of therapy after transplantation (days)	Dose of triazolopyrimidine (mg/kg)	Duration of therapy (days)	Mean tumor wt. (mg.)	Standard deviation (mg.)	Standard deviation of mean (mg.)	Average body weight (gm.) Beginning	End
42	Control	10				1,129	330	110	18.1	21.2
	Treated	10	1	50 once daily	13	309	95	32	17.8	18.1
	Treated	10	1	50 twice daily	13	90	47	16	18.8	16.9
45	Control	9	9*			238	81	29		
	Control	10				1,002	335	112	18.8	21.1
	Treated	10	9	50 once daily	5	620	159	53	18.3	19.7
	Treated	10	9	50 twice daily	5	403	152	51	20.0	19.7

* Animals sacrificed to determine mean tumor weight at initiation of therapy.

TABLE 6

EFFECT OF THE TRIAZOLOPYRIMIDINE ON LEUKEMIA 9417 IN AK MICE AND THE 6C3HED TUMOR IN C3H MICE

Exp. no.	Neoplasm	Group	No. of animals	Initiation of therapy after transplantation (days)	Dose of triazolopyrimidine (mg/kg)	Mean survival after tumor transplantation (days)	Standard deviation (days)	Standard deviation of mean (days)
8	9417	Control	12			12.8	1.54	0.46
		Treated	12	1	50 once daily	11.5	8.25	2.48
10	9417	Control	12			13.5	0.96	0.29
		Treated	12	1	12.5 once daily	17.7	4.52	1.36
14	9417	Control	9			13.1	1.56	0.55
		Treated	10	1	12.5 once daily	17.1	3.70	1.23
34	6C3HED	Control	9			15.6	5.50	1.95
		Treated	10	1	50 once daily	15.2	4.00	1.26

kemia or the lymphosarcoma. However, before these results are accepted as unequivocally negative, it must be emphasized that the number of animals studied is small. This was due to the difficulty of obtaining these mouse strains in quantity. For this reason, we wish to report these observations as a preliminary result which suggests that the triazolopyrimidine will be ineffective in altering the course of these two experimental neoplasms.

DISCUSSION

Table 7 summarizes the over-all effect of the triazolopyrimidine on a variety of experimental neoplasms which have been observed by Kidder and his associates, Burchenal *et al.* (1), and this group. With the exception of a single discrepancy, it would be justifiable to conclude from these experiments that the triazolopyrimidine has an inhibitory action on experimental carcinomas but not on the sarcoma and the leukemias. An exception to this generalization is the lymphatic leukemia which Kidder reported to be inhibited by the chemical substance. Before his observation can be accepted as valid, however, it must be recognized that Kidder reached his conclusion on the basis of a comparison of the peripheral blood count and "palpable tumor masses" of control and treated mice. The experience of chemotherapy in experimental leukemias indicates that the most important criterion for evaluating the significance of a chemical compound is survival time—all other observations being secondary.

TABLE 7

SUMMARY OF EFFECT OF THE TRIAZOLOPYRIMIDINE ON EXPERIMENTAL TUMORS

Tumor designation	Type of tumor	Inhibitory effect of triazolopyrimidine	Reference
Eo771	Adenocarcinoma of breast	positive	Kidder (3)
C3H spontaneous	Adenocarcinoma of breast	positive	"
Lymphoma 2	Lymphatic leukemia	positive	"
Leukemia Ak 4	Leukemia	negative	Burchenal (1)
755	Adenocarcinoma of breast	positive	Present report
RC	Adenocarcinoma of breast	positive	" "
Eo771	Adenocarcinoma of breast	positive	" "
Sarcoma 180	Undifferentiated sarcoma	negative	" "
9417	Leukemia	negative	" "
6C3HED	Lymphosarcoma	negative	" "
Brown-Pearce	Undifferentiated squamous-cell carcinoma	positive	" "

It is also apparent from the results which have been reported in this paper that the chemical pattern of all neoplastic cells is not uniform, inasmuch as the triazolopyrimidine fails to inhibit significantly several tumors.

In spite of the absence of a universal static action on all the tumors studied, it is of very real interest that triazolopyrimidine inhibits a variety of experimental tumors has been reported. Three transplantable adenocarcinomas of the breast in mice (755, RC, and Eo771) were effectively inhibited by the chemical agent, whether therapy was instituted shortly after transplantation or after the tumor had become well established; anterior chamber transplants of an undifferentiated squamous-cell carcinoma (Brown-Pearce) in rabbits' eyes were similarly controlled by the triazolopyrimidine. No demonstrable inhibitory effects of the chemical on an undifferentiated sarcoma (Sarcoma 180) could be detected, nor was there any apparent inhibition of an acute leukemia (9417) or lymphosarcoma (6C3HED) in preliminary experiments, with the dosage employed.

Using the changes in body weight during therapy as an over-all index of toxicity of the triazolopyrimidine, it was observed that the therapeutic activity of the drug against the tumors was not associated with significant toxicity to the host.

In spite of convincing evidence of tumor in-

of carcinomas without significant toxicity to the host. The fact that this chemical agent has such a favorable therapeutic index places the compound in an unique position among the drugs which have been studied in the field of experimental and clinical cancer chemotherapy. The evidence obtained from the experiments here reported lends strong support to Kidder's demonstration of a biochemical difference between the cells of a carcinoma and those of normal tissues. This stimulating fact kindles the hope that the triazolopyrimidine and its congeners may prove to be extremely useful tools for exploring the biology of neoplastic disease and thereby ultimately indicate chemical weapons which will specifically inhibit cancer in man.

SUMMARY

The action of the guanine analog, 5-amino-7-hydroxy-1*H*-*v*-triazolo (*d*) pyrimidine, on a variety

hibition, no regression of established neoplasms under therapy was observed, and the viability of inhibited cells was unimpaired, as indicated by their ability to reproduce normally on subsequent transplantation. With the exception of the Brown-Pearce carcinoma, no morphological changes in the tumors of treated animals could be detected.

The results presented in this report fail to confirm the hypothesis of a uniform chemical pattern in all neoplastic tissue. However, the evidence at hand suggests that the triazolopyrimidine distinguishes a biochemical difference between normal tissues and certain carcinomas.

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The Effect of 8-Azaguanine on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma and Melanoma in Animals*

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In recent years, one of the promising approaches to cancer chemotherapy has been the concentration of study on preparations believed likely to have anti-metabolite activity. Some of the compounds known to be anti-metabolites for microorganisms or for higher species have also shown adverse effects upon tumor tissues, e.g., desoxypyridoxine (9), anti-folic acids (2, 3, 5, 6, 11), anti-riboflavin (10) and the anti-purines, 2,6-diaminopurine (1) and "guanazolo"[1] (8-azaguanine) (4).

The observation that 8-azaguanine (5-amino-7-hydroxy-1*H*-*v*-triazolo [*d*] pyrimidine) temporarily inhibited the development of mammary adenocarcinoma E0 771 in C57 black mice, spontaneous breast cancers in C3H mice, and lymphoma 2 in strain A mice was considered evidence in favor of the uniformity of various cancer tissues with respect to this aspect of purine metabolism (4); however, the lack of inhibition of Sarcoma 180 by 8-azaguanine (7) is inconsistent with that concept. Such differences in the response of tumors to various chemotherapeutic agents may be welcomed, because of the increased opportunity offered to elucidate any peculiarities in tumor metabolism. The data reported below represent an attempt to confirm some of the observations of Kidder and his co-workers and to extend information on the spectrum of tumor inhibition by 8-azaguanine.

EXPERIMENTAL

In addition to adenocarcinoma E0 771 and spontaneous mammary cancer in C3H mice, the following tumors were used for this study: Crocker sarcoma 180, Lewis sarcoma T241, Harding-Passey melanoma, Wagner osteogenic sarcoma, and Patterson lymphosarcoma[2] in mice; and Flexner-Jobling carcinoma, sarcoma R39, and Walker carcino-sarcoma 256 in rats.

Subcutaneous implantations of tumors (small pieces of tumor, each measuring about 1.5 mm. cubed and weighing approximately 6 mg.) into healthy young animals (18–22-gm. mice; 100–125-gm. rats) were carried out by the usual trocar method—usually a single implant into the right axillary region. In general, the first intraperitoneal injection of the compound was given 1–7 days after tumor transplantation, and injections were continued for 8–20 days. The animals were given food (Purina Laboratory Chow) and water *ad libitum*.

In the present series of experiments, six samples of 8-azaguanine[3] were used. Sample B was a crude preparation. Sample C contained 60 per cent 8-azaxanthine, and samples D, E, F, and J were from 95 to 99 per cent pure.

Fresh suspensions of 8-azaguanine in 10 per cent gum acacia in saline were prepared once or twice weekly and kept in a refrigerator until used.

In every set of experiments, tumor-bearing animals were divided into two groups, one to be treated with 8-azaguanine and the other for controls. The progress of the tumors in the animals was recorded graphically by measuring them in two diameters with calipers every week.

* This work was supported from a grant by the Charles F. Kettering Foundation to the Wellcome Research Laboratories and by a grant to the Sloan-Kettering Institute from the American Cancer Society.

[1] The compound will be designated in this paper as 8-azaguanine. The name guanazolo is objectionable for several reasons, especially because of its similarity to guanazole, a long-established name for tetrahydro-3,5-diimino-1,2,4-triazole, cf. Chemical Abstracts.

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[2] The Wagner osteogenic sarcoma and the Patterson lymphosarcoma occurred spontaneously in the Sloan-Kettering colony of AK mice and will be described in detail later.

[3] In addition to samples from two of the authors, G. H. H. and L. F. C., we are greatly indebted to Dr. M. L. Crossley, of the Calco Division of the American Cyanamid Company, for the samples of 8-azaguanine used in these experiments. We are indebted to Dr. Aaron Bendich for the determination of purity by means of counter-current distribution technic.

The effect upon tumor growth was graded according to the following scheme:

++ = Marked inhibition; failed to grow or grew to approximately $\frac{1}{4}$ the average diameter of the controls (observation at 1–3 weeks).

+ = Moderate inhibition; tumors averaged from $\frac{1}{4}$ to $\frac{1}{2}$ the diameter of the controls.

± = Slight inhibition; the tumors grew to from $\frac{1}{2}$ to $\frac{3}{4}$ the diameter of the controls.

− = No effect; the tumors grew to $\frac{3}{4}$ or more the diameter of the controls.

Effect of 8-Azaguanine on mammary adenocarcinoma E0 771.—The adenocarcinomas in C57 black mice were treated with doses of 50–200 mg/kg of 8-azaguanine in one or two intraperitoneal injections daily, for from 8 to 20 consecutive days. The injections were begun 1 or 6 days after transplantation of the tumor. The results obtained with adenocarcinoma E0 771 are shown in Table 1.

TABLE 1

EFFECT OF 8-AZAGUANINE ON ADENOCARCINOMA EO 771 IN MICE*

Exp. no.	Sample of 8-aza-guanine	Amt. of 8-azaguanine mg/kg/day	No. of days† injected	Deaths from drug toxicity	Results of treatment	Remarks
1	E	50	13	0	++	Tumors in treated mice grew very slowly.
	E	50	8	0	−	Treatment started 6 days after implantation.
2	B	75	12	2	+	Six tumors grew slowly; 2 grew rapidly.
3	C	75	12	1	++	Five tumors grew very slowly; 4 grew slowly for 2 weeks.
	D	75	12	0	−	One tumor grew slowly; others grew rapidly.
	E	75	12	3	++	Eight tumors grew very slowly; others grew slowly for 3 weeks.
4	F	75	14	1	++	Eight tumors grew very slowly; others grew slowly for 2 weeks.
5	F	75	13	0	+	Five tumors grew very slowly; others grew slowly.
	F	75	8	0	−	Treatment started 6 days after implantation.
6	F	100	14	0	+	All tumors grew slowly for 2 weeks.
7	F	125	20	2	++	Tumors grew very slowly for 3 weeks.
8	J	125	10	3	++	All tumors grew very slowly for 3 weeks.
9	J	125	10	5	++	All tumors grew very slowly for 2 weeks.
10	J	175	10	9	++	Tumors grew very slowly for 2 weeks.
11	J	200	9	5	++	Tumors grew very slowly for 2 weeks.

* Ten tumor-bearing mice in each experimental group were matched with ten untreated controls. The tumors in the control group grew rapidly in every instance.

† Injections were started 1 day after tumor implantation, except where noted in remarks, when they were started 6 days later. Animals in experiment 2,8, and 10 were injected once daily; all others were injected twice daily.

It is clear that samples C, E, F, and J have a definite inhibitory effect on the growth of adenocarcinoma E0 771. However, they have not caused complete destruction of the tumor. When the injections were stopped, the small tumors grew normally and eventually caused the death of animals. Sample B showed a less inhibitory effect, and sample D had no effect. Typical examples of results obtained with samples C, D, and E are shown graphically in Figure 1.

Although 8-azaguanine showed a marked inhibitory effect upon the development of tumors when treatment was begun 24 hours after tumor implantation, it had practically no effect on the development of tumors if treatment was delayed until 6 days after transplantation (Table 1, experiments 1 and 5).

In two experiments, the influence of 8-azaguanine was evaluated by weighing the tumors 14 days after implantation. In the first group, ten animals were injected with 50 mg/kg/day of sample E in two injections on 13 consecutive days, starting 1 day after tumor implantation. Ten animals of the second group were treated similarly on 8 consecutive days, but daily injections of sample E were begun 6 days after implantation of the tumor tissues. Fourteen days after tumor implantation, the tumors of the treated and untreated animals were removed, measured, and weighed. The results obtained in this experiment and in a similar experiment performed with sample F are shown in Table 2. During the experimental period of 14 days, none of the animals died. In the seventh column of the table, the average size of the tumors is given by 3 diameters.

There is a striking difference in tumor size and weight between the two treated groups; in one group, treatment was started when the tumors were 1 day old and in the other when tumors were 6 days old. It is clear that the development of 6-day-old tumors was not noticeably affected by

the repeated injections of 8-azaguanine, for there was no significant difference in tumor weight between the treated and the control groups.

These results do not completely support the findings of Kidder and his co-workers (4). They reported a strong inhibitory effect upon the growth

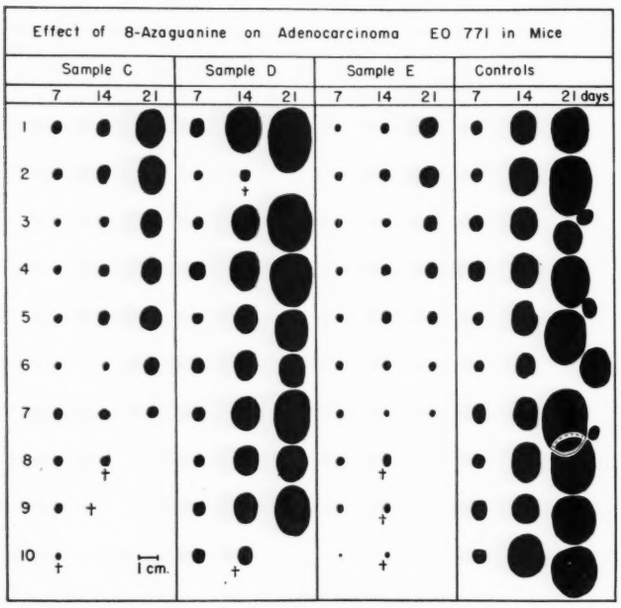

FIG. 1.—Repeated intraperitoneal injections of Samples C and E, 75 mg/kg of 8-azaguanine, caused a definite inhibitory effect upon the growth of mammary adenocarcinoma EO 771 in mice, but sample D had no effect (Table 1, Exp. 3).

of 6-day-old adenocarcinoma E0 771 in C57 black mice by repeated subcutaneous or intraperitoneal injections of 0.5 mg. of "guanazolo" twice daily (50 mg/kg for a 20-gm. mouse). The average size of their control tumors on the 14th day after tumor inoculation was 5.7 cm.^{3}, while "guanazolo"-treated tumors on the same day had an average size of 0.5 cm.^{3}, a ratio of approximately 11 to 1.

It is interesting to note that the control tumors in Kidder's experiments were much larger (5.7 cm.^{3}) than ours (1.3 cm.^{3}).[4] This difference is probably due to the difference in the amounts of tumor tissue injected. We found that some of our normal tumors grew rapidly and at the end of 14 days after transplantation measured 5 cm.^{3}. However, the average diameter of over 200 tumors, measured at the end of 14 days, was 1.9 × 1 5 × 0.9 cm., with a calcu'ated volume of 1.3 cm.^{3}.

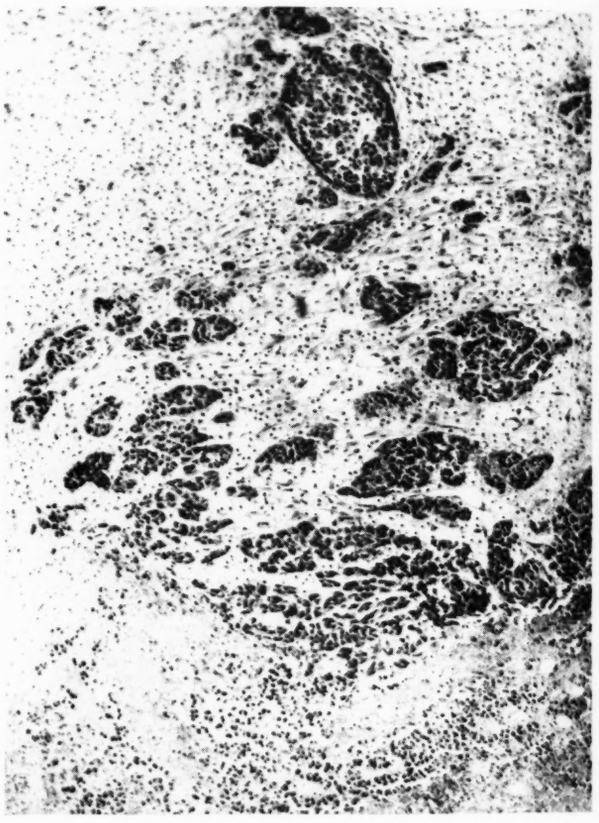

FIG. 2.—Adenocarcinoma EO 771 after treatment with 8-azaguanine (75 mg/kg for 12 days). Section shows small islands of active tumor surrounded by gelatinous edema and necrosis.

TABLE 2

SIZE AND WEIGHT OF 8-AZAGUANINE-TREATED 14-DAY-OLD ADENOCARCINOMA EO 771

Exp. no.	Sample	Age of tumors at 1st inj. (days)	Amt. 8-azaguanine mg/kg/day	No. of days injected	Average dimen. of tumors* (cm.)	Average wgt. of tumors* (gm.)
1	E	1	50	13	0.8×0.8×0.5	0.27
	E	6	50	8	1.5×1.3×0.8	1.28
	Controls				1.8×1.5×0.9	1.56
2	F	1	75	13	0.6×0.5×0.4	0.16
	F	6	75	8	1.2×1.1×0.6	0.66
	Controls				1.2×1.1×0.7	0.67

* Ten tumors were used in each determination.

When the tumors were allowed to grow for only 1 day before the injections were begun, inhibition of tumor growth was apparent in almost all instances. The tumors grew very slowly. At the end

[4] This volume was calculated on the basis of the formula for an ellipsoid which was considered to approximate the shape of the tumors.

of an experimental period of 14 days, the average tumor size was 0.17 cm.^{3} in the case of animals treated with sample E, and 0.06 cm.^{3} in the case of animals treated with sample F, while the controls were 1.3 cm.^{3}, and 0.48 cm.^{3}, respectively, a ratio of 8 to 1.

It is interesting to note that repeated intraperitoneal injections of 8-azaguanine into C57 black mice bearing adenocarcinoma E0 771 produced extensive gelatinous edema in the subcutaneous tissues and tissues of the thoracic and abdominal walls (observed at the end of 7 days). It was more pronounced on the right side, which received the tumor implantation and intra-peritoneal injections of the compound. This condition was generally accompanied by extensive hemorrhage. It also produced intraperitoneal hemorrhage and ascites. Histological examination of sections revealed small islands of active tumor surrounded by gelatinous edema and necrosis (Fig. 2). Extreme congestion and fine granules in the cytoplasm were observed in the liver. There was extensive congestion in the kidney. Reduction of lymphoid tissue was noted in the lymph nodes.

Effect of 8-Azaguanine on spontaneous mammary cancer.—Our attempts to confirm Kidder's findings of strong inhibition of spontaneous breast cancer in C3H mice with 8-azaguanine have been unsuccessful. The tumor-bearing animals were injected intraperitoneally with 50 mg/kg of sample E, 75 mg/kg of sample F, or 200 mg/kg of sample J daily in two injections for 4–22 days. This study included 25 C3H mice bearing 31 spontaneous mammary adenocarcinomas (Roscoe B. Jackson Memorial Laboratory) and 18 Swiss mice bearing 26 spontaneous mammary adenocarcinomas (Rockland Farms) in the treatment group and an equal number of tumor-bearing controls. The tumors measured from 8 to 15 mm. in diameter. The average body weight of treated mice was 38 gm.

The results of this experiment showed that daily doses of 50–75 mg/kg of 8-azaguanine—in two injections—had a slight inhibitory effect on the growth of spontaneous mammary cancers in mice,

TABLE 3

EFFECT ON VARIOUS MOUSE TUMORS

Exp. no.	No. of mice with tumor*	Amt. of 8-aza-guanine mg/kg/day†	No. of days injected	Deaths from drug toxicity	Results of treatment	Remarks
			Sarcoma 180			
1, 2, 3	30	75	10–13	0	−	Rapid growth.
4	10	100	12	2	±	Slight inhibition 1 week only.
5	10	125	12	1	±	Slight inhibition 1 week only.
6	10	175	8	4	+	Growth slow 1st week; thereafter more rapid.
7, 8	20	200	8–11	1	+	Growth slow 1st week; thereafter more rapid.
			Sarcoma T241			
9, 10	20	75	8–20	0	−	Rapid growth.
11	10	125	13	0	−	Rapid growth.
12	10	175	8	4	±	Slight inhibition 1 week only.
13	10	200	8	7	±	Slight inhibition 1 week only.
			Harding-Passey melanoma			
14	10	75	22	0	−	Same as controls; slow growth.
15	10	125	14	6	±	Slow growth, slightly less than controls.
16	10	200	10	9	±	Slow growth, slightly less than controls.
			Wagner-osteogenic sarcoma			
17, 18	20	75	18–20	0	− to ±	Tumors grew like controls in one exp. and slightly slower in another.
19	10	100	10	3	−	Rapid growth.
20	10	175	8	5	+	Growth inhibited definitely for 2 weeks.
20, 21, 22	30	200	4–8	26	+	Growth definitely inhibited; some animals died within 5 days.
			Patterson lymphosarcoma			
23	10	50	12	0	−	Rapid growth; no significant increase in survival time.
23, 24	20	75	12	0	−	Rapid growth; no significant increase in survival time.
25, 26	20	100	7–8	12	− to ±	Rapid growth; no increase to very slight increase in survival time.
27	10	175	8	5	−	Rapid growth; decrease in survival time.
25, 26, 28	30	200	7–9	22	+	Definite inhibition of tumor growth; no appreciable increase in survival time.

* In addition, an equal number of control animals were used in each experiment.

† The treatment was started 1 day after tumor implantation in every experiment except No. 14, in which treatment was delayed 4 days.

while daily doses of 200 mg/kg had a moderate inhibitory effect; however, at this level, many treated animals died. There was no complete destructive action on the spontaneous tumors.

Effect of 8-Azaguanine on Crocker mouse sarcoma 180, Lewis mouse sarcoma T241, Harding-Passey mouse melanoma, Wagner mouse osteogenic sarcoma, and Patterson mouse lymphosarcoma.—8-azaguanine was tested against a number of other types of mouse tumors, following the routine procedures already described. Sarcoma 180 and the melanoma were grown in albino mice of Rockland Farms strain; the osteogenic sarcoma and lymphosarcoma were grown in mice of AKm strain, while sarcoma T241 was grown in the same strain of C57 black mice in which it originated. The results obtained from these experiments are summarized in Table 3.

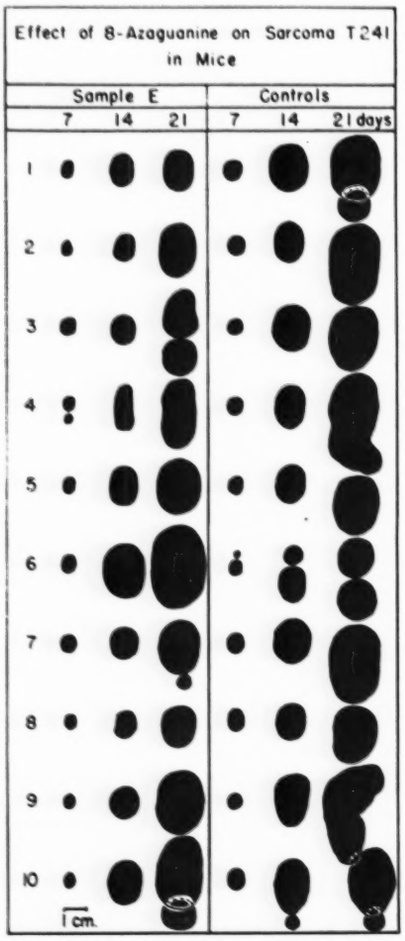

FIG. 3.—No inhibitory effect on the growth of sarcoma T241 in C57 black mice treated with 75 mg/kg of 8-azaguanine (sample E) for 20 days (Table 3, Exp. 9) is shown.

It is clear that the repeated injections of 50–75 mg/kg of 8-azaguanine caused no inhibition of the growth of Sarcoma 180. Inhibition was observed only at higher, toxic levels, in confirmation of the previous study in which no inhibition of Sarcoma 180 at tolerated doses (7) was found. The intraperitoneal injections of 8-azaguanine into albino mice bearing Sarcoma 180 produced a gelatinous edema in the subcutaneous tissues and tissues of the thoracic and abdominal walls. However, the degree of the edema was much less than it was in C57 black mice. The compound also produced ascites in albino mice.

The results with sarcoma T241 were also negative at tolerated doses, regardless of the more extensive gelatinous edema in the subcutaneous tissue around the tumor implants (Fig. 3). The slowly growing melanoma and the rapidly growing osteogenic sarcoma were not inhibited at nontoxic doses of the compound. At tolerated doses, 8-azaguanine neither inhibited the growth of the implanted Patterson lymphosarcoma nor prolonged significantly the survival of the treated animals beyond the controls. The treated animals died with extensive metastases in the spleen, lymph nodes, liver, and mesentery. At toxic levels of 175 and 200 mg/kg of 8-azaguanine, there was a moderate inhibitory effect on the primary tumor but no complete destructive action. Many of the treated animals (200 mg/kg) had no visible metastases at autopsy, but this may be because the animals died relatively soon, before metastases could develop.

Effect of 8-Azaguanine on Flexner-Jobling rat carcinoma, rat sarcoma R39, and Walker rat carcino-sarcoma 256.—Our studies with various transplantable mouse tumors have shown that 8-azaguanine adversely affects one type of tumor, but in other types of tumors it has no therapeutic value.

In a search for more striking effects, the study was extended to transplantable rat tumors, namely, the Flexner-Jobling carcinoma, Sarcoma R39 and Walker carcino-sarcoma 256. The grafts of these tumors grew well and reached a size of approximately 3.0 × 1.5 × 1.0 cm., 5.0 × 3.0 × 2.5 cm., and 4.5 × 2.5 × 1.0 cm. (diameters in 3 dimensions), respectively, at the end of 14 days after transplantation.

As in the case of mouse tumors, daily intraperitoneal injections of 75–200 mg/kg of 8-azaguanine were begun 1–4 days after tumor implantation. In most instances, the compound was given in two injections daily, for 8–16 consecutive days. In this study, samples E, F, and J were used, because they had a definite inhibitory effect on the growth of adenocarcinoma E0 771.

From the results summarized in Table 4, it is clear that repeated intraperitoneal injections of

8-azaguanine at all the levels, 75, 100, 125, and 200 mg/kg, had no inhibitory effect upon the growth of Flexner-Jobling carcinoma, Sarcoma R39, or Walker carcino-sarcoma 256 in rats. The progress of the tumor grafts in the treated animals was almost the same as that of the control tumors. Graphic illustrations of a number of more or less typical examples of the progress of tumor growth in treated and untreated animals show more clearly the lack of therapeutic value of 8-azaguanine in controlling these rat tumors (Fig. 4).

Albino rats (Sherman stock) tolerated the compound better than did Rockland albino mice or C57 black mice. No deaths occurred at the levels of 75 and 125 mg/kg of the compound. At the level of 200 mg/kg/day, 15 per cent of the rats died during the treatment.

It is interesting to note that in the tumor-bearing albino rats gelatinous edema or ascites was not observed.

DISCUSSION

The experimental data for the various tumors are summarized in Table 5. It is apparent that none of the rat tumors were inhibited with even the highest dose of 8-azaguanine. It can be seen, also, that mammary adenocarcinoma E0 771 is the only mouse tumor among those tested, including the spontaneous tumors, that is inhibited by 8-azaguanine at well-tolerated dose levels. The inhibition was observed when the treatment was started 24 hours after tumor implantation. There was little or no effect when treatment was delayed 6 days. In the absence of details on tumor implantation and measurement (4), it is not possible to account for the difference between the present results and those reported by others. (4).

An earlier study (7) revealed Sarcoma 180 as the first tumor found to be unaffected by adequate treatment with 8-azaguanine. The results of the present study indicate that Sarcoma 180, to the

TABLE 4

EFFECT OF 8-AZAGUANINE ON FLEXNER-JOBLING CARCINOMA, SARCOMA R39 AND WALKER CARCINO-SARCOMA 256 IN RATS

Exp. no.	Sample	No. of tumor	No. of rats with tumors	Age of tumors at 1st inject (days)	Amt. 8-Aza-guanine mg/kg/day	No. of days in-jected	8-Aza-guanine toxicity deaths	Complete tumor regres-sion (per cent)	Results of treat-ment	Remarks
1	Controls	FRC*	10					30		Three tumors regressed after growing for 2–3 weeks; others grew rapidly.
	F	"	10	4	75	16	1	20	—	Two tumors regressed after growing for 3 weeks; others grew rapidly.
2	Control	FRC	10					30		Three tumors regressed after growing for 1–4 weeks; 1 grew slowly; others grew rapidly.
	E	"	10	1	100	15	0	30	—	Three tumors regressed after growing for 1–3 weeks; others grew rapidly.
3	Control	FRC	10					20		Three tumors regressed after growing for 2–4 weeks; others grew rapidly.
	J	"	10	1	200	15	0	10	—	One tumor regressed after growing for 3 weeks; others grew rapidly.
4	Control	R39	5					0		Rapid growth.
	F	"	5	3	75	8	0	0	—	Tumor growth somewhat slow for 2 weeks; rapid growth thereafter.
5	Control	R39	5					0		Rapid growth.
	F	"	5	3	125	10	0	0	—	Rapid growth.
6	Control	R39	10					0		Two tumors grew slowly; others grew rapidly.
	J	R39	10	1	200	12	6	0	—	Four tumors grew rapidly.
7	Control	R39	10					10		One tumor regressed after growing for 2 weeks; others grew rapidly.
	J	R39	10	1	200	14	0	20	—	Two tumors regressed after growing for 1 week; one grew slowly; others grew rapidly.
8	Control	W256	10					10		One tumor regressed after growing for 1 week; others grew rapidly.
	F	"	10	1	75	14	0	0	—	Rapid growth.
9	Control	W256	10					0		Rapid growth.
	E	"	10	1	100	14	0	0	—	Rapid growth.
10	Control	W256	10					0		Rapid growth.
	J	"	10	1	200	14	0	0	—	One tumor grew slowly; others grew rapidly.

* FRC designates Flexner-Jobling rat carcinoma; R39 designates rat Sarcoma R39; W256 designates Walker rat carcino-sarcoma 256.

extent revealed by the lack of effect from 8-azaguanine, is not unique among tumors with respect to purine metabolism. The failure of 8-azaguanine to inhibit three types of rat tumors and seven different mouse tumors would appear to emphasize the statement that "it would be hazardous to assume that *all* malignant cells, unlike the normal, metabolize guanine, and therefore, will become inhibited by guanazolo" (4). Of considerable interest is the difference in the response of Sarcoma 180 and mammary adenocarcinoma E0 771 to 8-azaguanine. It has been noted that these two tumors are also affected differently by folic acid analogs (8). While such findings would appear to magnify the task of the cancer chemotherapist, they may facilitate studies on mechanisms of action of pertinent compounds. Thus, the fortunate response of E0 771 to 8-azaguanine provides a useful tool for learning more about the purine metabolism of certain abnormal cells.

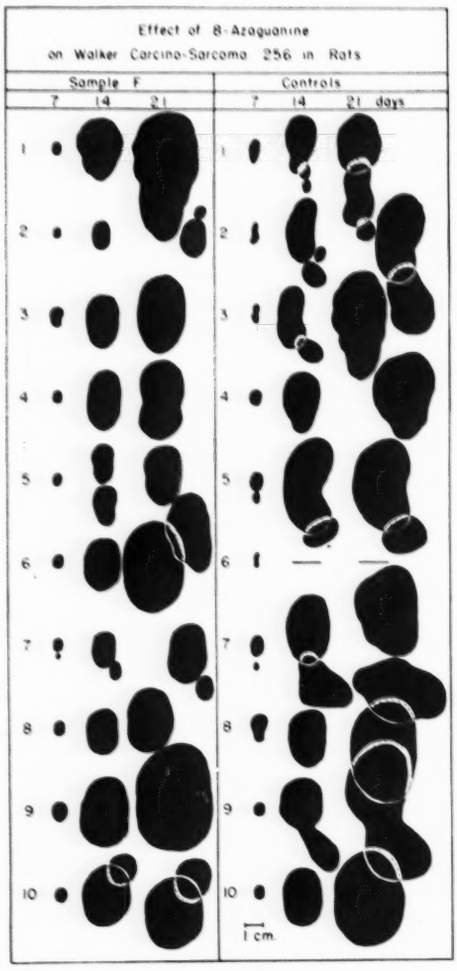

FIG. 4.—Repeated intraperitoneal injections of 8-azaguanine (sample F), 75 mg/kg for 14 consecutive days, caused no inhibitory effect upon the growth of Walker carcino-sarcoma 256 in rats (Table 4, Exp. 8).

The role of color and species' differences in the host were considered when mammary adenocarcinoma E0 771 in C57 black mice was found to be inhibited by 8-azaguanine. Sarcoma 180 was tested in C57 black mice, without inhibition from doses of 75–100 mg/kg/day of the compound. It is also clear that there was no effect on sarcoma T241 tested in C57 black mice. The lack of inhibition of Sarcoma 180 and sarcoma T241 in C57 black mice also suggests the lack of significant influence of the edema on the inhibition of adenocarcinoma E0 771. This is further indicated by the equal inhibition of bilateral implants of E0 771, regardless of the localization of the subcutaneous edema on one side of the animals.

SUMMARY

1. 8-azaguanine (5-amino-7-hydroxy-1*H*-*s*-triazolo [*d*] pyrimidine) had a definite inhibitory effect on the growth of mammary adenocarcinoma E0 771; however, it had no complete destructive action upon this tumor.

2. The inhibition was most apparent when the compound was administered early in the development of the tumor. There was little or no effect on adenocarcinoma E0 771 if treatment was delayed until 6 days after transplantation.

3. Spontaneous mammary cancers in C3H (Bar Harbor) and in Swiss mice (Rockland Farms) were apparently unaffected by repeated injections of 8-azaguanine.

4. The use of 8-azaguanine in the treatment of

TABLE 5

EFFECT OF 8-AZAGUANINE ON TUMORS IN ANIMALS*

	Mouse tumors						Rat tumors		
Amt. of compound mg/kg/day	Adeno-carcinoma E0 77	Sarcoma 180	L Sarcoma T241	H-P Melano-ma	W Osteo-genic sarcoma	P Lympho-sarcoma	F-J Carci-noma	Sarcoma R39	W Carcino sarcoma 256
50	++	−				−			
75	++	−	−	−	−	−	−	−	−
100		±		−	−	−	−		−
125	++	±	−	±				−	
175	++	+	±		+	±			
200	++	+	±	±	+	+	−	−	−

* − indicates no effect; ± indicates slight inhibition; + indicates moderate inhibition; ++ indicates marked inhibition.

mice bearing Sarcoma 180, sarcoma T241, Harding-Passey melanoma, Wagner osteogenic sarcoma, or Patterson lymphosarcoma, and of rats bearing Flexner-Jobling carcinoma, Sarcoma R39, or Walker carcino-sarcoma 256, produced neither an inhibitory nor a curative effect upon these tumors.

5. Repeated intraperitoneal injections of 8-azaguanine into C57 black mice bearing adenocarcinoma E0 771 produced extensive gelatinous edema in the subcutaneous tissues and tissues of the thoracic and abdominal walls, and intraperitoneal hemorrhage and ascites. These changes were less pronounced in albino mice and were absent in albino rats.

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Studies on the Effects of a Guanine Analog on Acute Lymphoid Leukemias of Mice*

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Roblin and associates (9) in 1945 synthesized the guanine analog, 5-amino-7-hydroxy-1*H*-*s*-triazolo (*d*) pyrimidine which has been referred to by Kidder *et al.* (6) in a subsequent study as guanazolo. It has been found that in bacteria (9) and in *Tetrahymena geleii* (7) competitive inhibition of growth results following the use of this substituted pyrimidine. Release or reversal of growth inhibition was accomplished by guanine in both organisms.

TABLE 1

EFFECT ON BODY WEIGHT, ADRENAL WEIGHT, AND THYMUS WEIGHT IN CAF_1 MICE INJECTED WITH GUANAZOLO*

Dosage (μg/gm) daily	Dosage (μg/gm) total	Per cent change body weight (range mean weights)	Adrenal weight† (per cent body weight)	Thymus weight (per cent body weight)
0	0	+1.1 (19.6–19.8)	.035	0.30
25	250	−4.3 (18.2–17.4)	.034	0.36
50	500	+1.5 (19.1–19.4)	.035	0.34
75	750	−1.6 (18.5–18.2)	.043	0.26
112.5	1125	+5.8 (17.0–18.0)	.041	0.13

* Nonleukemic CAF_1 females injected daily with guanazolo for 10 days. Control mice injected similarly with 0.01 N NaOH. Five mice in each group.

† Paired adrenal weights. Adrenal and thymus weights measured on torsion balance.

Retardation of the growth of a transplantable mammary adenocarcinoma and of spontaneous mammary cancers in mice was reported, following injections of guanazolo suspended in distilled H_2O. Data on the inhibitory effect of this compound on a transplantable acute lymphoid leukemia were not conclusive. Attempts at reversal of the guanazolo inhibition in mice by the use of guanine were unsuccessful (5).

Burchenal *et al.* (1) reported no inhibitory effect of three triazoles, including guanazolo, on survival time of mice bearing a transplantable lymphoid leukemia AK-4, although a purine derivative, 2,6 diaminopurine, which has been found to be an antagonist of pteroylglutamic acid and of adenine (4), caused a definite increase in survival time of mice bearing the same transplantable leukemia (2).

The effect of guanazolo[1] on two transplantable acute lymphoid leukemias in mice, leukemia L 1210 (strain dba) and Lymphoma 2 (strain A), and of a lymphosarcoma, Lymphoma 1 (strain A), has been investigated. Description of these lymphomas has been given previously (8). Guanazolo was given at the following dosage levels to leukemic mice: 12.5, 25, 37.5, 50, 75, and 112.5 μg/gm body weight. The compound in solution in 0.01 N NaOH was injected subcutaneously daily, the total number of injections depending upon the type of leukemia used and upon the condition of the animals. A 0.1 per cent solution was used for the 12.5, 25, and 50 μg/gm dosage levels, and a 0.133 per cent solution (100 mg/75 cc) was used for the 37.5, 75, and 112.5 μg/gm dosage levels. Fresh solutions were prepared each week.

In a preliminary experiment we have observed the effects of different dosage levels of guanazolo in normal, nonleukemic CAF_1 mice (♀ C × ♂ A cross). No demonstrable effect on body weight or on the blood picture (hemoglobin, total and differential leukocyte counts) was observed after ten daily subcutaneous injections at dosage levels 25–112.5 μg/gm body weight. At the higher dosage levels, 75 and 112.5 μg, definite adrenal hyperplasia and thymus involution were evident, however (Table 1).

Definite, regular, and reproducible increase in survival time was found in both acute lymphoid leukemias following administration of guanazolo at different dosage levels (Tables 2 and 3). A total of 156 dba mice in twelve different experiments

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[1] Obtained through the courtesy of American Cyanamid Co., Lederle Laboratories Div., Pearl River, N.Y.

TABLE 2

Effect of Guanazolo on Acute Lymphoid Leukemia L 1210*

Experiment	Compound	Number of mice†	Dosage (μg/gm) Daily	Dosage (μg/gm) Total	Survival time mean range (days)	Increase in survival time	Mean‡ wt. change (gm.)
	Guanazolo	4	50	400	13.7 (9–17)	52 per cent	+0.7
1	"	8	25	200	15.1 (12–17)	68 per cent	+1.8
	Controls§	11			9.0 (9. 0)		+1.8
	Guanazolo	6	50	550	13.7 (12–15)	51 per cent	+0.7
2	"	8	75	825	13.9 (13–16)	53 per cent	−0.3
	Controls	7			9.1 (9–10)		+2.2
	Guanazolo	5	112.5	1,125	10.8 (9–14)	3 per cent	+0.2
3	"	5	50	500	12.2 (8–15)	16 per cent	+1.2
	Controls	5			10.5 (9–12)		+2.2
	Guanazolo	8	25	225	12.0 (9–15)	30 per cent	+1.9
	"	8	50	450	13.0 (11–14)	41 per cent	+0.8
4a	"	8	75	675	13.4 (9–18)	45 per cent	+0.3
	Controls	5			9.2 (8–12)		+2.6
	Guanine HCl	5	375	3,750	9.6 (8–11)		+0.9
	Guanazolo‖	5	25	250	14.4 (14–15)	22 per cent	+0.5
4b	Controls	5			11.8 (10–14)		+1.1
	Guanazolo	5	25	300	14.0 (10–17)	52 per cent	+0.4
5	Controls	5			9.2 (9–10)		+1.8
	Guanazolo+		25	300			
	Guanine HCl	8	375	4,500	10.2 (9–11)		−0.5
	Guanazolo	8	25	150	10.0 (8–12)	25 per cent	+2.0
	"	8	37.5	225	10.9 (9–13)	36 per cent	−0.5
6	Controls	8			8.0 (7– 9)		+2.4
	Guanazolo+		25	150			
	Guanine HCl	8	375	2,250	10.0 (9–11)		+0.4
	"		37.5	225			
	"	8	375	2,250	9.1 (7–10)		−1.0
	Guanazolo	9	50	450	11.3 (11–12)	24 per cent	+1.0
	Controls	9			9.1 (8–12)		−0.5
	Guanazolo+		50	300			
7	Guanine HCl#	8	375	2,250	9.4 (7–12)		−1.4
	"		25	150			
		8	375	2,250	8.0 (7– 9)		−1.4
	"		25	150			
		8	375	2,250	9.1 (8–12)		−1.0
	Guanazolo	10	50	600	12.6 (11–15)	29 per cent	+0.2
8	"	10	75	765	12.6 (10–15)	29 per cent	−0.2
	Controls§	10			9.8 (9–11)		+0.4
	Guanazolo	12	12.5	100	10.0 (9–11)	11 per cent	−0.4
9	"	13	112.5	900	11.6 (8–14)	29 per cent	−2.8
	Controls	8			9.0 (9.0)		+0.2
	Guanazolo	10	50	400	10.5 (10–11)	21 per cent	+1.1
	"	9	75	600	11.0 (8–13)	27 per cent	−1.3
10	Controls	10			8.7 (8–11)		+1.7
	Guanazolo+		50	400			
	Guanine HCl#	10	375	3,000	9.4 (7–10)		−1.9
	Guanazolo	8	37.5	300	11.7 (10–13)	29 per cent	0.0
	"	8**	50	250	11.1 (9–12)	22 per cent	+0.5
11	Controls	8			9.1 (8–10)		+0.3
	Guanazolo+		37.5	300			
	Guanine HCl#	9	375	3,000	10.0 (9–11)		−2.8
	Guanazolo	5	25	300	13.8 (13–15)	50 per cent	+0.9
12	"	6	50	400	15.2 (11–16)	65 per cent	+0.4
	"	5	75	525	15.4 (11–15)	67 per cent	−0.3
	Controls§	5			9.2 (8–12)		+2.6

* All inoculations of leukemic cells, except where noted, done intraperitoneally with leukemic cell brei. Approximately 800,000 cells inoculated in 0.1 cc. volume.

† Strain dba mice, 15–20 gm. used throughout. Since a sex difference in response was not noted, sexes are combined.

‡ Weights obtained 8 days after beginning of experiment.

§ Controls injected with 0.01 N NaOH.

‖ Inoculations of leukemic cells done subcutaneously.

Guanine HCl suspended in 5 per cent gum arabic in saline. In all other experiments guanine suspended in peanut oil. Controls in experiments 10 and 11 received 5 per cent gum arabic in saline.

** Guanazolo administered 96 hours after inoculation of leukemic cells.

TABLE 3

Effect of Guanazolo on Acute Lymphoid Leukemia, Lymphoma 2*

Experiment	Compound	Number of mice	Dosage Daily	Dosage µg/gm Total	Survival time mean range (days)	Increase in survival time	Mean† weight change (gm.)
1	Guanazolo	7‡	50	600	15.4 (14–17)	37 per cent	+0.6
	"	7	75	750	16.3 (14–20)	44 per cent	−0.3
	Controls	7§			11.3 (11–12)		+1.8
2	Guanazolo	8	25	250	13.9 (11–21)	17 per cent	+0.9
	"	8	50	500	14.5 (12–19)	22 per cent	−0.7
	Controls	9§			11.9 (11–15)		+1.4
3	Guanazolo	8	37.5	375	13.9 (11–16)	42 per cent	+0.5
	"	8	75	750	15.5 (13–19)	58 per cent	−0.4
	Controls	6			9.8 (9–10)		+2.2
4	Guanazolo	10	25	250	12.9 (12–15)	11.2 per cent	−1.0
	"	11	50	500	14.2 (12–20)	23 per cent	−0.3
	"	11	75	750	14.0 (13–16)	21 per cent	−1.3
	Controls	8			11.6 (11–12)		+0.5
5	Guanazolo	8	25	225	11.9 (11–13)	17 per cent	+1.0
	"	8	50	400	12.4 (11–14)	22 per cent	−0.7
	Controls	15			10.2 (9–11)		+0.3

* All inoculations with leukemic cell brei done intraperitoneally. Approximately 800,000 leukemic cells inoculated in 0.1-cc volume.
† Weights obtained 10 days after beginning of experiments.
‡ CAF_1 mice 20–25 gm. body weight of mixed sexes used throughout.
§ Controls injected with 0.01 x NaOH.

TABLE 4

Summary of Results of Tables 2 and 3

Compounds used	Dosage (daily) (µg/gm)	Number of mice	Mean survival time—days	Increase in survival time
		Leukemia L-1210		
Guanazolo	25	34	12.9 (10.0–15.1)	41.7 per cent
"	37.5	16	11.3 (10.9–11.7)	24.2 per cent
"	50	66	12.4 (10.5–15.2)	36.3 per cent
"	75	40	13.0 (11.0–15.4)	42.9 per cent
Guanazolo+ Guanine HCl	25, 37.5, and 50 375	67	9.4 (8.0–10.2)	
Controls		91	9.1 (8.0–10.5)	
4-amino-N^{10}-methyl-PGA*	3	41	21.0 (14.4–26.6)	130.8 per cent
		Lymphoma 2		
Guanazolo	25	26	12.9 (11.9–13.9)	18.3 per cent
"	37.5	8	13.9 (11.0–16.0)	27.6 per cent
"	50	34	14.1 (12.4–15.4)	29.3 per cent
"	75	26	15.1 (14.0–16.3)	38.5 per cent
Controls		45	10.9 (9.8–11.9)	
4-amino-N^{10}-methyl-PGA*	3	16	14.4 (11.0–17.0)	32.1 per cent

* Injections done intraperitoneally every other day.

TABLE 5

Comparative Blood Studies of Strain DBA Mice Inoculated with Leukemia L 1210*

Compound	Number of mice	Hb. gm. per cent	Reticulocytes	wbc	Granulocytes	Agranulocytes	Blasts
Guanazolo (37.5 µg/gm)	5	11.7 (11.5–12.0)	0.1 per cent	42,700 (28,000– 52,000)	49.4	45.3	5.3
Guanazolo+ Guanine HCl (37.5 µg/gm+375 µg/gm)†	5	10.7 (9.5–12.0)	0.4 per cent	60,700 (38,250– 89,750)	32.7	50.7	16.6
Controls	5	12.3 (11.0–13.0)	2.0 per cent	119,600 (67,000–197,000)	39.0	35.7	25.3
4-Amino-N^{10}-methyl-PGA	3	13.3 (12.7–13.5)		11,800 (10,500– 13,000)	35	63	2.0

* Blood studies done 8 days after inoculation of leukemic cells.
† Guanine HCl in 5 per cent gum arabic in saline given orally daily.

and 94 CAF_1 mice in five different experiments were used for studies on survival time. Table 4 summarizes the results of the individual experiments and compares the effects obtained, using guanazolo, with the effect of 4-amino-N^{10}-methyl folic acid (amethopterin), the most effective of the folic acid antagonists in retarding leukemic growth in our experience. The antifolic compound produced a 130.8 per cent increase in survival time in dba mice inoculated with leukemia L 1210, compared with a 37.9 per cent increase with guanazolo. Guanazolo was as effective as 4-amino-N^{10}-methyl folic acid in increasing survival time in CAF_1 mice inoculated with Lymphoma 2.

Guanazolo was effective over a wider dosage range in dba mice bearing leukemia L 1210 than in strain A mice bearing Lymphoma 2. Increase in survival time was as great at the dosage level, 25 μg/gm body weight, as at the higher level, 75 μg/gm body weight, in strain dba mice with leukemia L 1210, whereas in strain A mice with Lymphoma 2 it is indicated that guanazolo becomes increasingly effective with an increase in dosage. A definite toxicity was apparent in dba leukemic mice at dosage levels of 112.5 μg/gm body weight. Death of a small percentage of mice at this dosage level occurred before death of control leukemic mice, although typical changes characteristic of the folic acid deficiency syndrome, viz., hypoplasia of bone marrow, edema of the intestinal tract with associated desquamation, and diarrhea were not observed. Toxic symptoms were also apparent at this dosage level in normal, nonleukemic CAF_1 mice (Table 1). The discrepancy between these results and those of Kidder *et al.* (6), who stated that they observed no toxic effects of the guanazolo, may be explained by the difference in dosage levels, since their material was used in suspension and ours in solution.

Table 5 shows the effect on the blood picture in dba leukemic mice injected with guanazolo. Control animals developed a florid leukemia with a severe leukocytosis, and 25 per cent blast forms were found in the peripheral blood. Although a moderately high leukocyte count was found in guanazolo-treated mice at the 8-day level, relatively few blast cells were present. The inhibitory effect of the folic acid antagonist, 4-amino-N^{10}-methyl folic acid, on leukemic dba mice was found to be more pronounced 8 days after inoculation of leukemic cells, and the blood picture closely resembled that of the normal nonleukemic mouse.

Definite arrest of the leukemic process was evident in histologic sections of the spleen, liver, and lymph nodes. Moderate infiltration of leukemic cells into these organs was found, in contrast to the extensive infiltration in control mice. The marked reduction or complete absence of leukemic infiltration seen in leukemic mice given parenteral 4-amino folic acid (8) was not found in any of the mice examined in this study.

Marked reduction in the rate of growth of leukemic cells of acute lymphoid leukemia L 1210 inoculated subcutaneously was observed 8 days after inoculation (6 days after the beginning of parenteral administration of 50 μg/gm guanazolo). At this time, the local mass of lymphoma tissue weighed 566.2 mg. (five mice), while the mean weight of lymphoma tissue from guanazolo-treated mice was 213.4 mg. (five mice). It is pertinent to note that guanazolo at the same dosage level was effective in adrenalectomized mice. The mean weight of the subcutaneous lymphoma mass of adrenalectomized mice was 168.6 mg. (five mice).

No evidence of necrotic changes have been observed in lymphoma tissue grown in the subcutaneous regions or in leukemic cells infiltrating other organs.

No effect on survival time of CAF_1 mice bearing a lymphosarcoma, Lymphoma 1, or on the rate of growth of lymphosarcoma tissue was noted following daily subcutaneous injections of guanazolo at the dosage level 50 μg/gm. The survival time for controls was 39.7 days and for guanazolo-injected mice (total dose 463 μg/gm body weight) was 32.3 days (25 mice in each group).

In an attempt to arrive at some understanding of the mode of action of guanazolo in the inhibition of the growth of leukemic cells, release of inhibition was sought by the use of guanine HCl. Subcutaneous injections of guanine HCl in suspension in peanut oil or in 5 per cent gum arabic in saline were commenced 24 hours after inoculation of leukemic cells (24 hours prior to parenteral guanazolo treatment) and continued daily 4 hours before injection of the guanine analog. The dose used was 10–15 times by weight that of the analog (375 μg/gm body weight given in 0.2 cc. daily). This was reported to be the ratio of metabolite to inhibitor required to release completely the inhibition of guanazolo in *Tetrahymena* (7).

It can be seen, by reference to the individual experiments in Table 2 or in the summary in Table 4, that, judged on survival time, a release of the antileukemic effect of guanazolo is obtained. The survival time of dba mice bearing acute lymphoid leukemia L 1210 is essentially that of the untreated leukemic controls (9.4 days compared with 9.1 days). The gross and microscopic appearance of the leukemic process in these animals was similar to that seen in the leukemic control mice. Release of inhibition, as judged by the peripheral blood

picture, however, was only partial (see Table 5), although the leukocyte count was definitely higher, and the number of blast cells found approached that in the dba leukemic mouse. It should be noted that slight loss of weight was encountered in each experiment where metabolite and inhibitor both were given, probably because of the appearance of sterile abscesses in some of the mice at the site of inoculation of guanine HCl. In experiments now in progress, however, apparent release of inhibition is obtained without loss of weight, following oral administration of guanine HCl, and it is indicated that lower ratios of metabolite to inhibitor are effective.

Release of the anti-leukemic effect has not yet been accomplished by the use of pteroyltriglutamic acid or adenine sulfate.

It would seem from the available data that a relative deficiency of guanine is established by parenteral administration of the guanine analog, guanazolo, and that leukemic cells require a more abundant supply of guanine than do normal cells of the lymphoid series. A parallel situation seems to occur in leukemic mice given the folic acid antagonists, 4-amino folic acid and 4-amino-N^{10}-methyl folic acid, where the leukemic process may be controlled by an apparent slight deficiency of folic acid without seriously impairing the processes for normal cell life. The anti-leukemic effects of both of these folic acid antagonists have been blocked by folic acid or its derivatives (8, 3).

In general, it has been found that similar anti-leukemic effects have been shown by the antifolic compounds and by 2,6 diaminopurine and the guanine analog guanazolo. Whether or not the same locus of action is characteristic of all will be revealed by more complete reversal and hematologic studies.

SUMMARY

1. A definite, regular, and reproducible inhibition of two transplantable acute lymphoid leukemias in mice resulted from parenteral administration of the guanine analog, guanazolo.

2. No effect of this compound on a transplantable lymphosarcoma was observed.

3. Apparent release of inhibition by the use of guanine HCl is recorded.

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The Effect of Adrenal Grafting on Transplanted Lymphatic Leukemia in Rats*

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It is a well established fact that the adrenal cortical hormone exerts a depressing influence on the lymphoid tissue. A previous report from this laboratory showed that removal of the adrenals renders a rat far more susceptible to a transplantable lymphatic leukemia (1) and that treatment with the cortical hormone increases resistance to the disease (2). In the present investigation, the effect of auto- and homoplastic grafts of the adrenals on the resistance of rats to leukemia has been determined.

EXPERIMENTS

The rats used for these experiments were from a subline of the Wistar Institute strain and were approximately 8 weeks old. Inoculations were made about 2 weeks after the operative procedure.

GROUP 1.—These experiments were designed to test the effect of autografting. The adrenals of 49 rats were removed, cut in half, and the four halves reimplanted in the abdominal cavity. This proved to be ineffective in that 91.8 per cent of such animals proved susceptible to transplanted leukemia, while the 30 controls gave 80 per cent positive results.

GROUP 2.—In this group of experiments 47 rats were adrenalectomized, and one adrenal was returned to the abdominal cavity, and the other grafted into the spleen. The 50 control rats gave 94 per cent leukemia, while the grafted ones gave 85.1 per cent, a difference not mathematically significant.

GROUP 3.—The 241 rats used in this group of experiments were treated in the following way:

A.—Seventeen rats were subjected to adrenalectomy and 2 weeks later inoculated intraperitoneally with a suspension of leukemia cells. These rats proved susceptible in 100 per cent.

B.—The adrenals were removed from 65 rats, each adrenal was cut in half, and the halves were reintroduced into the abdominal cavity, with the addition of two adrenals from another rat. Ten days to 2 weeks later these animals were inoculated with leukemia cells. Only 26.1 per cent proved susceptible.

C.—Two adrenals were halved and both inoculated into the abdomen of each of 73 intact rats. Ten days to 2 weeks later the animals were inoculated with leukemia. Of these, 57.5 per cent proved susceptible, as compared with an 82.5 per cent susceptibility among the 86 controls.

GROUP 4.—These experiments were planned to show the effect of subcutaneous introduction of two additional adrenals into 34 intact rats, as compared with the susceptibility of 33 controls. The adrenal-grafted animals were rendered definitely more resistant, showing only 44.1 per cent susceptibility, as compared with 87.8 per cent in the controls. This result indicated that adrenals grafted subcutaneously are just as effective as the intra-abdominal grafts tested in group 3.

The results from the above experiments are brought together in Table 1. It will be seen from the first experiment that removal and reimplantation of the adrenals in the abdominal cavity does not affect resistance. The results are not modified when one of the grafts is introduced into the spleen where active growth of the adrenal grafts has been noted. Simple adrenalectomy markedly reduced resistance—a finding which confirms an earlier observation (1). The removal of the adrenals and the reimplantation in the abdomen with the addition of two adrenals from other rats resulted in very definite augmentation of resistance. While the introduction of two adrenals into the abdominal cavity of intact rats increased susceptibility to a significant degree, it was not increased to the extent observed in the foregoing group. The additional adrenals implanted subcutaneously gave results comparable to those with the grafts in the abdominal cavity.

DISCUSSION

The interpretation of the reported results is not entirely clear. Certainly, a definite increase of resistance to a transplanted lymphatic leukemia re-

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sulted from the implantation of two extra adrenals, whether these were added to either an intact rat or one that had undergone adrenalectomy and autografting. Autografting alone, into either the abdominal cavity or the abdominal cavity and spleen, had little or no effect; but autografting combined with homotransplantation had the most pronounced effect in inducing resistance of any of the procedures used. The figures were significantly different from those obtained from intact rats with adrenals grafted in the abdomen but not from intact rats with adrenals grafted subcutaneously. However, it is doubtful whether the greater effectiveness of combined auto- and homografting should be emphasized. In general, reduction in leukemia incidence may be attributed to the presence of additional adrenal glands, presumably through increased hormonal control. This is in line with our previous experiments with adrenal cortical extracts and preparations of pituitary adrenotropic hormones. It is known that a degree of resistance to this transplanted leukemia may be induced by the injection of normal homologous living cells (3–5), but it is believed that the number of cells in the adrenal homografts is insufficient to induce this nonspecific type of resistance (6). In judging the degree of effectiveness of the various procedures, it should be noted that not only quantitative hormonal relationships but also the phenomena of transplantation are involved.

The idea suggests itself that different degrees of incompatibility among grafted adrenals and host, or other uncontrolled variables, may enter the picture. Jaffe (7) demonstrated that autoplastic grafts of adrenals in the abdominal wall take and grow to a fairly large size. Even small grafts in adrenalectomized animals maintain life, and larger ones enable such animals to remain in good condition indefinitely. Homoplastic grafts take, but they degenerate after a few months. More recently, Everett (8) found that autografts of adrenals are successful, but only about 25 per cent of the grafts among litter mates take, and homografts among nonlitter mates rarely succeed. While there was no information in our experiments on the relation of the rats to each other, it is judged that the stock is genetically fairly uniform, because it shows such a high susceptibility to transplants of a tumor arising in the stock. In any case, there appeared to be a high rate of takes of the homoplastic grafts in our tests. The chief point of interest seems to be the increase in resistance to leukemia that accompanies an increase in adrenal tissue.

TABLE 1

EFFECT OF ADRENAL GRAFTING ON LYMPHATIC LEUKEMIA IN RATS

	No. of rats	No. with leukemia	Per cent leukemia	Average days survival
Group 1:				
Adrenalectomized and autograft of glands returned to abdomen	49	45	91.8	9.3
Intact controls	30	24	80.0	9.9
Group 2:				
Adrenalectomized—autograft, one in abdomen and one in spleen	47	40	85.1	8.7
Intact controls	50	47	94.0	10.7
Group 3:				
A. Adrenalectomized	17	17	100.0	7.8
B. Adrenalectomized—one and two additional adrenals from another rat grafted in abdomen	65	17	26.1	10.3
C. Intact rats—two adrenals grafted in abdomen	73	42	57.5	11.2
D. Intact controls	86	71	82.5	9.7
Group 4:				
Intact rats—2 adrenals grafted subcutaneously	34	15	44.1	12.0
Intact controls	33	29	87.8	8.7

SUMMARY

Rats in which the adrenals were removed and reimplanted intraperitoneally, with two additional adrenals from another rat, showed a definite increased resistance (73.9 per cent) to a transplanted lymphatic leukemia over that of control animals (17.5 per cent). The removal and reimplantation of the adrenals, one in the abdominal cavity and one in the spleen, without the addition of homoplastic adrenals, failed to induce any significant increase in resistance. The introduction of two homoplastic adrenals into intact rats reduced susceptibility to inoculated leukemia to 57.5 per cent, as compared to 82.5 per cent for the controls; and in a second

experiment to 44.1 per cent, as compared to 87.8 in the controls. The simple removal of the adrenals resulted in a 100 per cent susceptibility to leukemia, a finding which confirms an earlier observation.

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Effect of a Carcinogenic Azo Dye on Liver Cell Structure
Isolation of Nuclei and Cytoplasmic Granules*

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INTRODUCTION

Livers of rats fed certain azo dyes undergo a series of generalized histological and chemical changes culminating in malignant growth. Such livers provide favorable material for study of the mechanism of formation of cancer cells.

Previous investigators have identified particular structures in the liver cell that are concerned in the action of 4-dimethylaminoazobenzene (4-DAB) and its 3′-methyl derivative 3′-Me-4-DAB, a more active carcinogen. Loss of nucleic acid and protein from the isolated large granules of the hepatic cell cytoplasm during feeding of these dyes has been reported (6, 18). Administration of azo dyes also gives rise to unusually large liver cell nuclei (12) and causes an increase in the liver content of desoxypentosenucleic acid, a nuclear component (6, 9, 13, 18). The magnitude of these changes appears related to the rapidity of the carcinogenic process (18).

During 4-DAB carcinogenesis, certain chemical changes appear to parallel the precancerous histological changes (12, 14, 15), and similar correlations have been made by one group of workers (19) in studies with other carcinogenic azo dyes. The present study was concerned with the behavior of certain anatomical units of liver cells during the different stages of cancer induction by 3′-Me-4-DAB. Precancerous lesions were studied histologically, while nuclei and cytoplasmic granules were isolated for direct determination of several structural components—i.e., proteins and nucleic acids. Specific effects of the carcinogen's action were singled out by comparison with pair-fed control animals. At the same time, observations were made on the relation between the sex and the nutritional status of the animals and the structure of their liver cells.

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METHODS

Adult albino rats of the Sprague-Dawley strain were maintained on a basal semi-synthetic ration, previously described (9), with or without the addition of 0.06 per cent 3′-Me-4-DAB. The carcinogenic diet was fed *ad libitum*, and the average food intake was approximately 15 gm. per day. The controls were limited to the same intake as that given the azo-dye-fed rats. Female rats, after 4 and 12 weeks on carcinogenic and control diets, and males, after 3, 9, and 12 weeks on these diets, were deprived of food for 12 hours and sacrificed.

Livers of three rats, azo-dye-fed or control, were obtained at the same time. Under ether anesthesia, livers were perfused with cold physiological saline solution. Small portions of the liver were taken for histological studies, and the remainder was frozen in physiological saline at −5° C. for 24–48 hours.

Livers, after thawing, were homogenized in a sucrose medium by passing them once or twice through a Potter tissue grinder. Small strands of connective tissue which would not pass through the grinder were discarded. The resulting liver suspension was diluted with more of the sucrose medium, so that each ml. of homogenate contained 0.1 gm. of fresh liver.

The sucrose fractionating medium, modified from that employed by Price, Miller, and Miller (17) was of the following composition:

Sucrose	0.88 M
NaCl	0.14 M
Phosphate buffer pH 7.2	0.01 M

The nuclei were sedimented in a small angle-head centrifuge in a field approximately 500 × *g* for 45 minutes. Fifteen ml. of the homogenate were centrifuged in a 50-ml. tube, the supernate decanted, and a second 15-ml. sample layered over the sediment and centrifuged in the same way. After the second supernate was decanted, the nuclei were shaken up in 10 ml. of the sucrose medium and resedimented. The combined supernates and

washings were made up to 50 ml. with sucrose medium, and four 10-ml. samples were pipetted into 50-ml. centrifuge tubes. These were centrifuged for 15 minutes in an angle-head Servall supercentrifuge, in a field 20,000 × *g*, to sediment the large granules or mitochondria (10). The large granules in each tube were shaken up in 5 ml. of sucrose medium and recentrifuged as before.

The supernates and washings obtained after isolating the large granules were made up to 100 ml. with sucrose medium, and four 10-ml. samples were pipetted into 50-ml. centrifuge tubes. These were centrifuged for 4 hours in the Servall centrifuge, in a field 20,000 × *g*. The sediment was composed of small granules or microsomes (2), and the remaining fluid was designated the supernatant fraction. The above procedures were carried out in rooms maintained below 5° C. Centrifugations were carried out in a room at −5° C. For sedimentation of small granules, the centrifuge was refrigerated, so that the temperature within the tubes did not rise above 5° C.

The procedure differed materially from that employed by Price, Miller, and Miller (17), in that the livers were frozen and thawed before use and were suspended in a medium containing NaCl at "physiological" concentration. In a trial of the effect of these factors, using normal rat liver, almost identical amounts of protein and nucleic acid were obtained in the large granule fraction, with or without freezing, and with or without use of sodium chloride in the sucrose medium. Microscopically, the large granules prepared by the modified technic were not swollen and had the same appearance as in tissue sections. They resembled those isolated from a nonionic sucrose medium by Hogeboom, Schneider, and Pallade (10).

Nitrogen was determined by the micro-Kjeldahl procedures of Chibnall, Rees, and Williams (1). Previous work had indicated that more than 90 per cent of the total nitrogen of both precancerous and control livers is protein nitrogen (9). For nucleic acid determinations, protein was precipitated with 10 per cent trichloroacetic acid in the cold room and washed four times with cold 5 per cent trichloroacetic acid. Multiple washings were found necessary to remove sucrose. Nucleic acids were then extracted by heating for 20 minutes at 90° C. in 5 per cent trichloroacetic acid, and pentose and desoxypentose were determined by reactions with orcinol and diphenylamine, respectively, as employed by Schneider (21). For pentose, the color developed with the orcinol reagent was read at 663 mμ in a Beckman or Coleman spectrophotometer, and a correction for sucrose applied after reading at 420 mμ. For desoxypentose, the color developed with the Dische reagent was read at 594 mμ.

The analytical values, corresponding to protein components, were expressed on the basis of an amount of fresh liver containing 2.25 gm. of total nitrogen (i.e., 100 gm. of fresh liver, after correction to nitrogen content of 2.25 per cent). This was desirable because of the variation encountered, in different experimental groups, in nitrogen content of the whole homogenates (1.6–2.3 per cent of the fresh liver weight), which were attributed to variations in content of moisture and nonprotein solids.

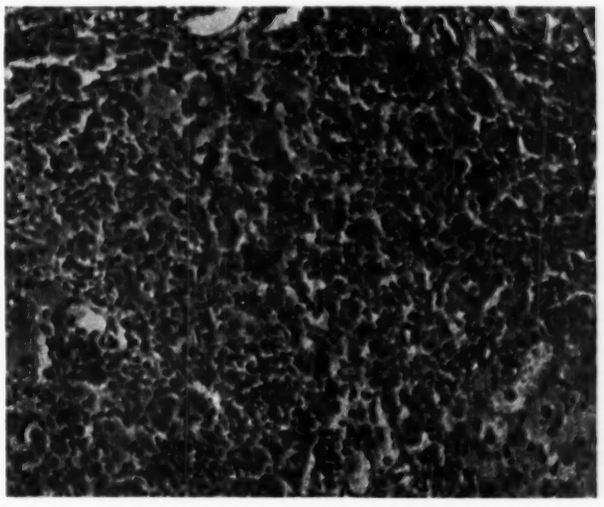

FIG. 1.—Mass of proliferating bile ducts from liver of male rat fed 3′-Me-DAB for 12 weeks. Hematoxylin and eosin. ×320.

RESULTS

In animals fed the carcinogen, nodular enlargement of the liver appeared between 6 and 9 weeks. Hepatomas were identifiable in nearly all animals after 12 weeks on the carcinogenic diets. No definite changes were noted either grossly or microscopically in livers of control animals during the feeding period.

In livers of animals fed the carcinogen, the most prominent alteration was proliferation of bile duct epithelial cells, which formed tubular structures that invaded the liver parenchyma (Fig. 1). Parenchymal cells also showed hyperplasia and eventual swelling and hypertrophy (Fig. 2). This process obliterated the normal liver sinusoid pattern shown in Figure 3. The histological changes were similar to those caused by the less active carcinogen, 4-DAB, except for the more rapid proliferation of bile duct cells. Further studies, using special histo-

logical technics, are in progress and will be reported later.[1]

Nuclei.—The desoxypentosenucleic acid content of the nuclear fraction prepared from livers of animals fed the carcinogen was consistently greater than that of controls, as shown in Table 1.

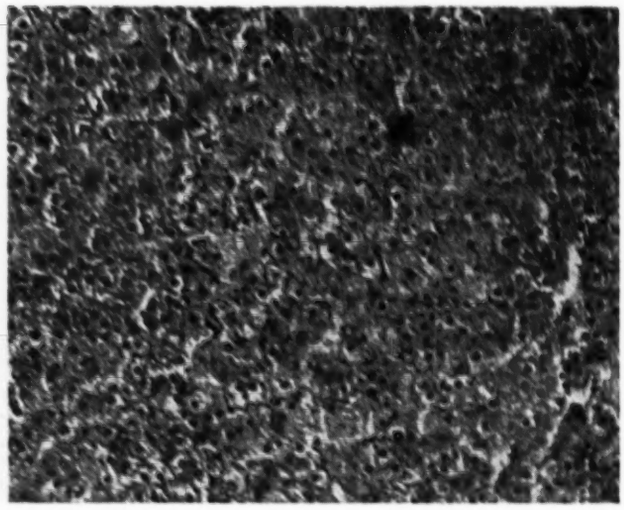

FIG. 2.—Parenchymal region of liver shown in Fig. 1. Hematoxylin and eosin. ×320.

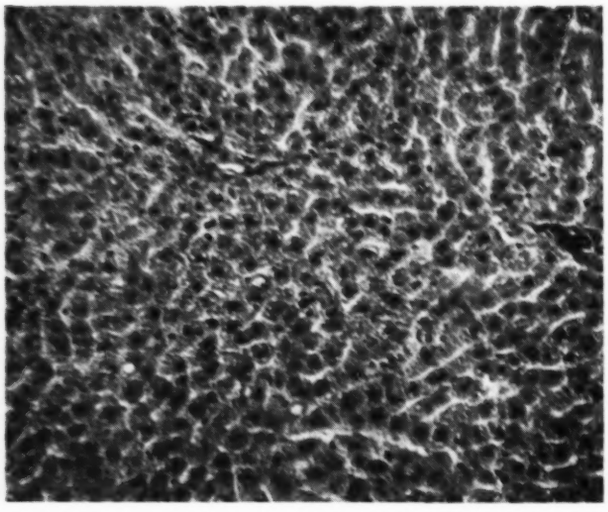

FIG. 3.—Liver of male rat fed basal diet 4 weeks, showing hepatic cell chords and sinusoids. Hematoxylin and eosin. ×320.

Histologically, in animals fed the carcinogen, the increased desoxypentosenucleic acid content could be correlated with increased numbers of nuclei in a given volume of tissue. However, in later stages, after 9 or 12 weeks of feeding the carcinogen, nuclear density was actually decreased in large areas, as compared to that of control livers (Fig. 2).

Nuclei contained all the desoxypentosenucleic acid, in livers of animals fed the carcinogen, as well as in controls. Cytoplasmic fractions did not give a true reading for desoxypentose, although their nucleic acid extracts, after mixing with Dische's diphenylamine reagent, gave a pale green color, with an absorption spectrum lacking the characteristic maximum in the neighborhood of 594 mμ. This reaction, unlike that with desoxypentosenucleic acid, took place rapidly at room temperature and was not obtained with fresh preparations of the diphenylamine reagent.

Higher values were found for desoxypentosenucleic acid and for total nitrogen in the nuclear fraction of females than in that of males. In male rats fed the carcinogen, the nitrogen content of the nuclear fraction was increased, as compared to that of controls, but this effect was not seen in females.

TABLE 1

DESOXYPENTOSENUCLEIC ACID AND NITROGEN OF NUCLEI OF CARCINOGEN-FED AND CONTROL RATS

Values in milligrams of total nitrogen or of desoxypentose nucleic acid, for an amount of fresh liver containing 2.25 gm. of nitrogen before fractionation.

Sex	Time on diets, weeks	Desoxypentose nucleic acid: Carcinogen-fed	Desoxypentose nucleic acid: Pair-fed controls	Nitrogen: Carcinogen-fed	Nitrogen: Pair-fed controls
Female	4	479	372	460	460
	12	566	406	521	535
Male	3			277	292
	9	354	264	392	210
	12	322	238	446	251

Microscopic examination revealed that the nuclear fractions were composed of intact nuclei like those seen in tissue sections. However, the preparation was contaminated with a few intact cells and with slender strands of debris attached to the nuclei. This cytoplasmic contamination appeared to have less relative significance in the values for nuclear nitrogen than in those for the pentose nucleic acid of this fraction, which varied from 5 to 20 mg., expressed as pentose, per 100 grams of liver. Little significance could be attached to these values, especially in view of the difficulty of estimating pentosenucleic acid accurately by a pentose determination in the presence of relatively large amounts of desoxypentosenucleic acid.

Cytoplasmic fractions.—The large-granule fraction contained practically all the rod-like struc-

[1] We are indebted to Dr. Howard Richardson of the Department of Pathology, University of Oregon Medical School, Portland, Oregon, under whose supervision much of this histological work was done. We also wish to thank Dr. Hadley Kirkman of the Department of Anatomy of Stanford University, for examining some of our slides.

tures ("mitochondria") demonstrable in liver cell cytoplasm in sections stained with iron hematoxylin. No microscopic structures could be found in the small granules and supernatant fractions.

Values for the nucleic acid and total nitrogen of these fractions are given in Table 2. When the carcinogen was administered, a reduction was noted in most cases in the protein and nucleic acid of the large-granule fraction.

Relative loss of nucleic acid from small granules, caused by feeding the carcinogen, was found only in females after 4 weeks. Variations in the small granule and supernatant fractions of controls were larger, in general, than differences between comparable values for precancerous and

Definite increases in nuclear density have not been noted in histological studies of the liver changes caused by the less active carcinogens, 4-DAB (8, 12, 14–16), *o*-aminoazotoluene (20), and N-acetylaminofluorene (5). However, Masayama and Yokoyama (13) were able to elicit rapid increases in desoxypentose nucleic acid content of rat livers with 4-DAB. It is interesting that these workers fed the less active carcinogen in a relatively deficient diet and were able to produce tumors in approximately the same time in which they were produced with the diet employed in the present study. Intense mitotic stimulation may be a necessary accompaniment of rapid carcinogenic action by azo dyes.

TABLE 2

DISTRIBUTION OF NUCLEIC ACID AND NITROGEN IN LIVER CELL CYTOPLASM OF CARCINOGEN-FED AND CONTROL RATS

Values for pentose nucleic acid (RNA) expressed as milligrams of pentose, for an amount of fresh liver (approximately 100 gm.) containing 2.25 gm. of nitrogen before fractionation. Values for nitrogen (N) in milligrams, for the same quantity of liver.

Fraction	Sex and time on diets							
	Female				Male			
	4 weeks		12 weeks		3 weeks		9 weeks	
	RNA	N	RNA	N	RNA	N	RNA	N
Large granules								
Precancerous	11.8	460	40.6	458	23.7	462	22.0	401
Control	24.2	690	35.3	461	34.9	560	31.3	615
Small granules								
Precancerous	8.6	214	14.5	87		143	16.5	155
Control	20.9	297	18.2	79		150	17.5	160
Supernatant								
Precancerous	99.5	1,110	90.0	1,100		1,080	93.8	1,130
Control	71.5	935	100.8	1,150		935	89.5	1,150
Total of fractions								
Precancerous	120	1,780	145	1,650		1,690	132	1,690
Control	117	1,920	154	1,690		1,650	138	1,930
Whole liver minus nuclei								
Precancerous	110	1,790	145	1,729		1,973	121	1,858
Control	112	1,790	150	1,715		1,958	124	2,040

control tissue. The nitrogen content of the small granules of females appeared reduced by prolonged maintenance on the restricted diets. Values for nitrogen and nucleic acid of the supernatant fraction were relatively constant in the different groups of animals.

DISCUSSION

The increased content of nucleic acid and nitrogen in liver-cell nuclei of animals fed the carcinogen may be explained either on the basis of increased numbers of nuclei in a given portion of liver or on the basis of increased content of these materials in individual nuclei. The histological observations were more consistent with the former explanation. The carcinogen evidently acted as a mitotic stimulant, resulting in increased nuclear density in the affected tissue.

The finding of reduced nucleic acid and nitrogen content of the large-granule fraction, in animals fed the carcinogen, is in agreement with the report of Price, Miller, Miller, and Weber (18). This confirmation was obtained, even though the technics employed in the two studies differed sufficiently to give results that disagreed in other respects, with regard to the distribution of protein and nucleic acid in the cytoplasm. This is evident from the fact that these workers found more of each component in the large and small granules, and less of each component in the supernatant fraction, than were found in the present study. This comparison emphasizes the consistency with which cancer induction by azo dyes is associated with this disappearance of large-granule material.

The analytical studies are paralleled by Opie's histological observation (15) of loss of basophilic

material (pentosenucleic acid) from large granules in the cytoplasm of the liver cells of rats fed 4-DAB. This author noted increased cytoplasmic basophilia in certain foci of livers in late stages of the precancerous process (15). It would be difficult to detect such localized phenomena by analyses of fractions of homogenates of whole liver.

Results of analyses of isolated cell components also agree with previous histological studies in emphasizing the importance of various physiological variables in determining the intracellular composition of rat livers. Thus, the relative size of nuclei is affected by the age of the animals (12) as well as by their sex. The size, shape, and number of the large granules of the cytoplasm vary according to the length of a fasting period following a standard meal (7).

The effect of carcinogens on the structure of liver cells must therefore be interpreted in relation to such factors as sex hormones and the nutritional state of the cells. Such considerations may help explain many of the phenomena of azo dye carcinogenesis which have been described. At present, it appears that a fundamental precancerous change is mitotic stimulation, accompanied by accumulation of desoxypentosenucleic acid (6, 9). Since this process is associated with disorganization of the biliary and vascular systems of the liver, some cytoplasmic changes which have been noted during carcinogenesis may depend on interference with the blood supply of the parenchymal cells or with the drainage of bile from them. Therefore, the cytoplasmic fraction of the livers may be affected secondarily by mitotic stimulation. This concept would relate the precancerous liver changes, caused by feeding azo dyes, to the hyperplastic precancerous lesions found in other tissues, clinically (11), and in experimental animals (3, 4).

SUMMARY

1. The structure of liver cells was studied in male and female rats fed the carcinogen, 3′-Me-4-dimethylaminoazobenzene, and in pair-fed controls. Histological observations were correlated with results of analyses of isolated nuclei, large granules, small granules, and remaining cytoplasm. These fractions were prepared by a modified method of differential centrifugation in solutions containing sucrose. Experience with the modified medium, containing NaCl, indicated that the success of the method of Hogeboom *et al.* in isolating "mitochondria" in their original form does not depend on use of a nonionic medium.

2. In confirmation of previous reports, administration of the carcinogen caused increase of desoxypentosenucleic acid and total nitrogen in the nuclear fractions, and loss of nucleic acid and protein from the large granules of the cytoplasm. Increased nuclear density, associated with precancerous proliferation in animals fed the carcinogen, contributed to increased content of the components of nuclei in the livers.

3. Female rats had more nucleic acid and nitrogen in the nuclear fraction than did males. Irregular changes were encountered in the composition of the cytoplasm in animals of different sexes in different nutritional states.

4. Changes affecting the nuclei of liver cells during administration of carcinogenic azo dyes appear to be of fundamental importance, as in other types of precancerous lesions.

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Announcement

Group Travel to the Fifth International Cancer Congress

Arrangements are being completed whereby a number of physicians and investigators may travel together as a group to the Fifth International Cancer Congress to be held in Paris from July 17 to 22, 1950, through the co-operation of Youth Argosy, Inc. This is an educational, nonprofit, membership organization whose purpose is to provide travel opportunities for worthy students of slender means, so that they may enjoy the benfits of foreign study and travel. After consultation with officials of this organization, travel to the Fifth International Cancer Congress meets the requirements.

The transportation charge by air, via a chartered airline with the Youth Argosy, Inc., will be $375.00 *round trip*. The flight will leave the United States on July 14 or 15 and will arrive the following day in Luxemburg. Transportation may be obtained as a group from Luxemburg to Paris at the cost of approximately $3.10 third class, $4.34 second class, or $6.62 first class.

The flight will return from Luxemburg a few days after the completion of the International Radiological Congress, which is being held in London the following week.

Wives can be accommodated in limited numbers, depending upon the number of physicians and investigators going as a group. Overseas travel insurance will be provided at a group rate.

Travel by ship may also be obtained, but at the present time arrangements may be completed only on a ship to sail from Quebec on July 1, to arrive in London on July 10. While the rates for ship travel ($328.00 round trip) will be somewhat less than those by air (accommodations will be dormitory style), taking into consideration tipping and transportation from New York to the embarkation point and from London to Paris and return, the total costs by ship and by air will be approximately the same.

The approximate sailing date from Le Havre will be about August 14.

To reserve space for this group, a $20.00 deposit should be sent to Dr. Brewster S. Miller, Assistant Director, Professional Education Section, American Cancer Society, Inc., 47 Beaver Street, New York 4, New York; this should be sent as soon as possible to insure the July 14 or 15 air travel dates. Checks or money orders should be made out to the Youth Argosy, Inc. As soon as the names of a group of 50 people going to the Congress are in his hands, confirmation of air departure dates may be obtained.

One-half of the total transportation charge, less the amount of the above deposit already remitted, shall be due and payable on March 27. The remaining one-half of the transportation charge shall be due and payable on April 24. For reservations made after May 1, a delayed-payment fee of $10.00 will be charged.